SUPPRESSORS (*scs1-scs7*) OF *CSG2*, A GENE REQUIRED BY *S. CEREVISIAE* FOR GROWTH IN MEDIA CONTAINING 10mM Ca^{2+}, IDENTIFY GENES REQUIRED FOR SPHINGOLIPID BIOSYNTHESIS

1994

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ABSTRACT

Title of Dissertation: Suppressors (*scs1-scs7*) of *CSG2*, a Gene Required by *Saccharomyces cerevisiae* for Growth in Media Containing 10 mM Ca^{2+}, Identify Genes Required for Sphingolipid Biosynthesis.

Chun Zhao, Doctor of Philosophy, 1994.

Dissertation directed by: Dr. Teresa M. Dunn, Ph.D.
Associate Professor
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The gene, *CSG2*, is required for growth of *S. cerevisiae* cells in media containing 10 mM Ca^{2+}. It was cloned, sequenced and found to be an integral membrane protein with nine potential membrane spanning domains and a potential Ca^{2+}-binding site (EF-hand) predicted to be in a cytosolic loop.

To further investigate the function of CSG2p, a collection of mutants (**S**uppressors of **C**a^{2+} **S**ensitivity) that suppress the *csg2Δ* Ca^{2+}-sensitive phenotype was selected at 37°C on YPD + 100 mM Ca^{2+} plates. These suppressors are recessive and they fall into seven complementation groups (*scs1-scs7*). All members of the *scs1* and *scs2* groups simultaneously acquire a Ca^{2+} (>10 mM) requirement for growth whereas wild-type cells grow with only trace amounts of Ca^{2+}. This Ca^{2+}-requiring phenotype is not rescued by Sr^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, or Ba^{2+}. The *SCS1* gene was cloned by complementation of the *scs1* Ca^{2+}-requiring phenotype. The

sequence of the *SCS1* gene shows that it encodes a 561 amino acid protein which is homologous to a family of pyridoxal phosphate enzymes that catalyze the transfer of an acyl group from an acyl-CoA donor to the α-carbon of an amino acid. The *SCS1* gene was disrupted by one step gene replacement. This *SCS1* null mutant lacks serine palmitoyltransferase activity and requires phytosphingosine or dihydrosphinganine for growth indicating that *SCS1* encodes serine palmitoyltransferase. This enzyme catalyzes the first committed step in sphingolipid biosynthesis (palmitoyl-CoA + serine -> 3-ketosphinganine + CoA + CO_2). The other *scs* mutants as well as the *csg2* null mutant also have altered sphingolipid metabolism. The data indicate that sphingolipid metabolism in yeast is either regulated by Ca^{2+} and/or is required for Ca^{2+} homeostasis.

Suppressors (*scs1-scs7*) of *CSG2*, a Gene Required by *Saccharomyces Cerevisiae* for Growth in Media Containing 10 mM Ca^{2+}, Identify Genes Required for Sphingolipid Biosynthesis

By

Chun Zhao

Dissertation submitted to the Faculty of the Department of Biochemistry Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1994

DEDICATION

To

My Father, Mother and My Teachers ...

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CONTENTS

ABBREVIATIONS

ATP; adenosine 5'-triphosphate

Ba^{2+}; barium ion

BIO; biotin

BRL; bethesda research laboratory

Ca^{2+}; calcium ion

$CaCl_2$; calcium chloride

CDC; cell division cycle

$CHCl_3$; chloroform

CH_3OH; methanol

CH_3CH_2OH; ethanol

$CH_3CH_2OCH_2CH_3$; ether

Co^{2+}; cobalt ion

CoA; coenzyme A

CSG; calcium sensitive growth

dATP; deoxyadenosine 5'-triphosphate

DNA; deoxyribonucleic acid

EDTA; ethylenediaminetetraacetic acid

EGTA; ethylene glycolbis(ß-aminoethyl ether) N, N, N', N'-tetraacetic acid

EMS; methanesulfonic acid ethyl ester

ER; endoplasmic reticulum

GDP; guanosine 5'-diphosphate

H^+; proton ion

HEM; heme

HEPES; N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

H_2O; water

HCl; hydrochloride acid

IPC; inositolphosphorylphytoceramide

K^+; potassium ion

KBL; α-amino-ß-ketobutyrate coezyme A ligase

KCl; potassium chloride

kD; kilodalton

3-KDS; 3-ketodihydrosphingosine

LCB; long chain base

LEU; leucine gene

MES; 2-(N-morpholinolethanesulfonic acid)

Mg^{2+}; magnesium ion

$MgSO_4$; magnesium sulfate

MIPC; mannoseinositolphosphorylphytoceramide

$M(IP)_2C$; mannose(inositolphosphoryl)$_2$phytoceramide

Mn^{2+}; manganese ion

mRNA; messenger ribonucleic acid

NaN_3; sodium azide

NH_4OH; ammonium acetate

Ni^{2+}; nidkel ion

ORF; open reading frame

PIPES; peperazine-N, N'-bis(2-ethanesulfonic acid)

PLP; pyridoxal phosphate

PMC; plasma membrane Ca^{2+}-ATPase

PMR; plasma membrane ATPase related genes

RNA; ribonucleic acid

SERCA; sarco/endoplasmic reticulum

SCS; suppressors of calcium sensitivity

SOC; subunit II of cytochrome c oxidase

SPT; serine palmitoyltransferase

Sr^{2+}; strontium ion

Tris; Tris(hydroxymethyl)aminomethane

TrpE; a gene encodes for anthranilate synthease

URA; uracil gene

Zn^{2+}; zinc ion

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CHAPTER ONE

General Introduction

Calcium is a regulatory ion:

1. Cytosolic calcium is tightly regulated.

All cells maintain a low cytosolic Ca^{2+} level by actively pumping Ca^{2+} out of the cell or into organelles. The cytosolic Ca^{2+} concentration in unactivated cells is typically 0.1 μM, several orders of magnitude less than that in the extracellular milieu. Because of this large Ca^{2+} gradient, even the transient opening of Ca^{2+}-channels in the plasma membrane or in an intracellular membrane can cause dramatic changes in the cytosolic Ca^{2+} concentration. This is one reason that Ca^{2+} is capable of acting as an intracellular messenger.

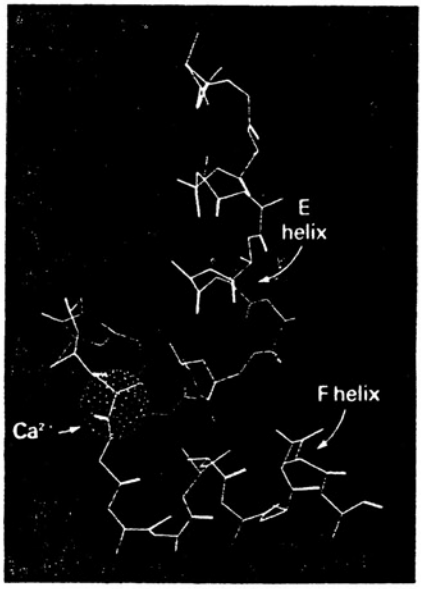
2. Some proteins bind Ca^{2+} with high affinity and selectivity.

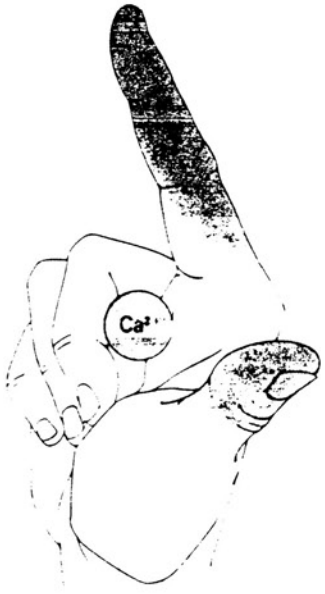
A second reason calcium is well suited to act as a regulatory ion is that proteins can form high affinity Ca^{2+} binding sites that are very specific for Ca^{2+} [Stryer]. Ca^{2+} binds to both negatively charged oxygens (*e.g.* the oxygen from side chains of glutamate and aspartate) and uncharged oxygens (*e.g.* the oxygen in main-chain carbonyls). Ca^{2+} coordinates six to eight oxygen ligands. This enables Ca^{2+} to cross-link different segments of a protein to induce large conformational changes. Furthermore, the binding of Ca^{2+} to oxygen has a high selectivity. Mg^{2+}, a potential competitor, and the only divalent cation found in millimolar concentration cannot substitute for Ca^{2+} because it does not have an appreciable affinity for uncharged

oxygen atoms. Another important difference between these ions is that Mg^{2+} prefers to form small and symmetric coordination shells, whereas Ca^{2+} can form asymmetric complexes with larger radii. Therefore, Ca^{2+} will bind well to irregularly shaped crevices in proteins and can be selected over Mg^{2+} even when the latter is a thousand-fold more abundant inside the cell.

The EF hand, a structural domain that binds specifically to Ca^{2+}, has been identified by X-ray crystallographic studies of calcium-binding proteins. Parvalbumin, a 12-kDa Ca^{2+}-binding protein in carp muscle, has two similar Ca^{2+}-binding sites, which are formed by a helix-loop-helix. Eight oxygen atoms are coordinated to each Ca^{2+}. Six coordinating oxygens are from three aspartates and one glutamate, one main-chain carbonyl oxygen, and one oxygen of a bound water molecule. Helices E and F in this protein form one Ca^{2+}-binding site. They are positioned at right-angles like the forefinger and thumb of the right hand (Figure 1). The Ca^{2+}-binding site is within the loop between these helices. This structural motif was named the "EF hand" by Kretsinger [Kretsinger and Nockolds, 1973; Moews and Kretsinger, 1974] who suggested that the two Ca^{2+}-binding sites of parvalbumin were formed by duplication of a primordial gene encoding a calcium-binding loop. He also found homologous amino acid sequences between parvalbumin and troponin C and suggested that troponin C also contains EF hands [Kretsinger and Barry, 1975] which has since been proved by X-ray analyses. Now it is appreciated that the EF hand is a recurring motif in calcium-binding proteins.

Figure 1. The Ca^{2+}-binding motif that is formed by a helix-loop-helix unit is called an EF hand. Helix E runs from the tip to the bottom of the forefinger. The flexed middle finger corresponds to the EF Ca^{2+}-binding loop. Helix F runs to the end of the thumb. [Stryer, 1991]





EF
hand

Ca^{2+} also functions as a regulatory ion in *S. cerevisiae*:

It has been difficult to demonstrate that *Saccharomyces cerevisiae* requires Ca^{2+} for vegetative growth using Ca^{2+}-depleted rich media (YPD-Ca^{2+}) or Ca^{2+}-free synthetic media. Nonetheless, results from numerous lines of investigation indicate that Ca^{2+} plays an important regulatory role in *S. cerevisiae* [Davis and Thorner, 1986; Iida *et al.*, 1990a]. For instance, protein phosphorylation and cell cycle are known to be controlled by Ca^{2+}. Many proteins known to be regulated (directly or indirectly) by Ca^{2+} are present in *S. cerevisiae* including calmodulin [Davis *et al.*, 1986], calmodulin-dependent protein kinase [Ohya *et al.*, 1991a], calmodulin-regulated protein phosphatase [Cyert *et al.*, 1991], kex2 protease [Mizuno *et al.*, 1989], trehalase [Neves *et al.*, 1992], glycogen phosphorylase [Francois *et al.*, 1988], phospholipase C [Yoko-o *et al.*, 1993], vacuolar K^+ channel [Bertl and Slaymen, 1990], and protein kinase C [Levin *et al.*, 1990]. Furthermore, numerous Ca^{2+}-sensitive [Ohya *et al.*, 1986a; Ohya *et al.*, 1986b; Ohya *et al.*, 1991b] and Ca^{2+}-dependent [Ohya *et al.*, 1984; Schmitt *et al.*, 1988] mutants have been isolated.

Three Ca^{2+} transporters have been identified in yeast: (1) A low-affinity H^+/Ca^{2+} antiport activity is present in isolated vacuole membranes [Ohsumi and Anraku, 1983]. The exchanger is driven by the proton gradient across the vacuolar membrane; (2) The *PMR1* protein which is a member of the sarco/endoplasmic reticulum (SERCA) family of Ca^{2+}-ATPases [Serrano, 1991] is thought to transport Ca^{2+} into Golgi complex [Rudoph *et al.*, 1989; Antebi and Fink, 1992]; (3) A plasma-membrane type Ca^{2+}-ATPase, *PMC1*, is reported to transport Ca^{2+} from the

cytoplasm into the vacuole [Cunningham and Fink, 1994]. It is a member of the plasma membrane Ca^{2+}-ATPase family. The low cytosolic Ca^{2+} concentration (0.1 μM) is maintained by either pumping Ca^{2+} out of the cell (through the Ca^{2+} ATPase on the plasma membrane) or transporting Ca^{2+} into the organelles (through the Ca^{2+} ATPase on the Golgi membrane or the Ca^{2+} ATPase and the H^+/Ca^{2+} exchanger on the vacuolar membrane).

Experimental rationale and approach to identify proteins that are required for Ca^{2+} homeostasis in yeast.

Since *S. cerevisiae* has become an experimental model system for investigating many eukaryotic processes that are controlled by Ca^{2+} [Davis *et al.*, 1986; Iida *et al.*, 1990b], it is of interest to identify the genes and proteins that are important in regulating the cytosolic Ca^{2+} concentration (100-200 nM [Iida *et al.*, 1990b; Dunn *et al.*, 1994]). Cytosolic Ca^{2+} is apparently regulated by the active transport of Ca^{2+} from the cytosol into internal organelles such as the Golgi or vacuole, and out of the cell through the plasma membrane [Eilam, 1982; Ohsumi and Anraku, 1983; Rudolph *et al.*, 1989]. For Ca^{2+} to serve as a regulatory signal, mechanisms should also exist for Ca^{2+} to flow into the cytosol in response to the appropriate stimuli.

One approach toward identifying the genes (and the proteins) that are important in regulating the cytosolic Ca^{2+} is to isolate mutants that lose the ability to grow in medium containing high Ca^{2+} concentrations, identify those mutants with alterations in the regulation of cellular Ca^{2+}, and then clone and characterize the

genes that complement the mutations. Anraku and co-workers [Ohya *et al.*, 1986b] have generated a collection of Ca^{2+}-sensitive mutants that represent 18 complementation groups. Nine of the genes are required for vacuolar morphogenesis or encode subunits of the vacuolar proton ATPase [Ohya *et al.*, 1991b] suggesting that functional vacuoles are required for Ca^{2+} homeostasis. One of the genes is allelic to *CDC24* [Miyamoto *et al.*, 1987]. We have also generated a collection of Ca^{2+}-sensitive mutants. The mutagenesis and prescreen growth was done in media containing 1 mM ethylene glycolbis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and the primary screen for Ca^{2+}-sensitive mutants were done at 37°C to allow identification of temperature-sensitive, Ca^{2+}-sensitive mutants. To eliminate vacuolar mutants, only the mutants that were sensitive to 50 mM Ca^{2+} but resistant to 50 mM Sr^{2+} were selected because the vacuolar defective Ca^{2+}-sensitive mutants are also Sr^{2+} sensitive.

CHAPTER TWO

The Ca^{2+}-Sensitive Mutant *csg2* Identifies a Gene That Is Required for Growth in High Ca^{2+}

INTRODUCTION:

As mentioned above, 50% of the complementation groups in Ca^{2+}-sensitive mutant collection isolated in Anraku's laboratory [Ohya *et al.*, 1991b] are defective in vacuolar morphogenesis or acidification. Since the vacuolar Ca^{2+}/H^{+} exchanger also transports Sr^{2+}, defects in the vacuole would be expected to cause Sr^{2+} as well as Ca^{2+} sensitivity [Ohsumi and Anraku, 1983]. Thus a collection of Ca^{2+}-sensitive mutants which are resistant to Sr^{2+} was isolated in an attempt to identify novel Ca^{2+} transport genes not related to vacuole function. Nineteen *csg* (**C**alcium **S**ensitive **G**rowth) mutants that lost the ability to grow in 100 mM Ca^{2+} (but remained insensitive to 50 mM Sr^{2+}) were identified in a screen of approximately 60,000 mutagenized yeast colonies. The *csg* mutants were identified as members of the *csg1* group (7 members), the *csg2* group (4 members) and as 8 independent isolates [Beeler *et al.*, 1994]. Cells carrying mutations in the *CSG2* gene grow normally in low Ca^{2+} medium, but cease growth when the Ca^{2+} concentration is above 10 mM. The *csg2* mutant cells accumulate much higher levels of Ca^{2+} in a compartment that is exchangeable with extracellular Ca^{2+}, whereas the nonexchangeable Ca^{2+} pool which predominates in wild-type cells is not influenced. Sr^{2+} influx is not increased in the *csg2* mutant cells. Mg^{2+} decreases the amount of Ca^{2+} in the nonexchangeable pool

without influencing the *csg2*-induced exchangeable Ca^{2+} pool [Beeler *et al.*, 1994]. The data indicate that the *csg2* mutation causes a selective increase in Ca^{2+} accumulation into a pool which is distinct from the vacuolar pool. The *CSG2* protein consists of 410 amino acids with nine putative transmembrane segments, four potential sites for N-linked glycosylation, and contains a sequence homology to the EF-hand Ca^{2+} binding site.

MATERIAL & METHODS:

Strains: The yeast strains used in this study were DBY947: *Matα ade2-101 ura 3-52*, TDY2040: *Mata ade2-101 ura3-52 csg2::LEU2*$^+$ *trp1Δ leu2Δ*, TDY2039: *Mata ade2-101 ura3-52 trp1Δ leu2Δ*, TDY2038: *Matα lys2 csg2::LEU2*$^+$ *ura3-52 trp1Δ leu2Δ*, TDY2037: *Matα lys2 ura3-52 trp1Δ leu2Δ*, and TDY821: *Mata/α ura3-52/ura3-52 lys2/+ +/ade2-101 trp1Δ/trp1Δ leu2Δ/leu2Δ can*^{R}*/can*^{S}, or were derived from them by standard crosses.

Plasmids: plasmids pRS316 and PRS306 have been described [Sikorski and Hieter, 1989]. M13mp18 and mp19 phage were used to generate single-stranded template DNA for sequence determination.

Media: Yeast media, YPD (rich medium), YP (YPD medium without glucose) and SD (synthetic medium) and *E. coli* medium, LB (rich medium) [Sherman *et al.*, 1974; Miller, 1972] were used.

Chemicals and Enzymes: Restriction endonucleases were obtained from either New England Biolabs Inc. or Gibco BRL Life Technologies. T4 DNA ligase was supplied by Gibco BRL Life Technologies. Ethanol was purchased from Pharmco. Guanidinium thiocyanate was supplied by Fluka Biochemika. The α-[^{32}P]dATP was purchased from DuPont-New England Nuclear. The 12CA5 antibody was supplied by Babco (Berckley Antibody Company). All other chemicals were obtained from Sigma.

Nucleic acid manipulation:

1. The preparation of high molecular weight DNA was based upon the method of Struhl *et al* [1979].

2. Plasmid DNA was prepared from *Escherichia coli* by a modification of the method of Holmes and Quigley [1981]. The isopropanol precipitated DNA was resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and incubated on ice for 15 min in the presence of 2.1 M of ammonium acetate (NH_4Ac). The debris was removed by centrifugation at 12,000 g for 5 min at 4°C. Then the DNA was precipitated by adding two volumes of cold ethanol. The RNA was digested by RNase (50 μg/ml) and the contaminating proteins were removed by phenol extraction. The purified plasmid DNA was precipitated by cold ethanol.

3. The yeast cells were cultivated, treated and transformed by the method of Ito *et al.* [1983] with a modification that 100 μg of sonicated salmon testes DNA (Sigma: #D-7656) was routinely added as carrier DNA to each transformation reaction.

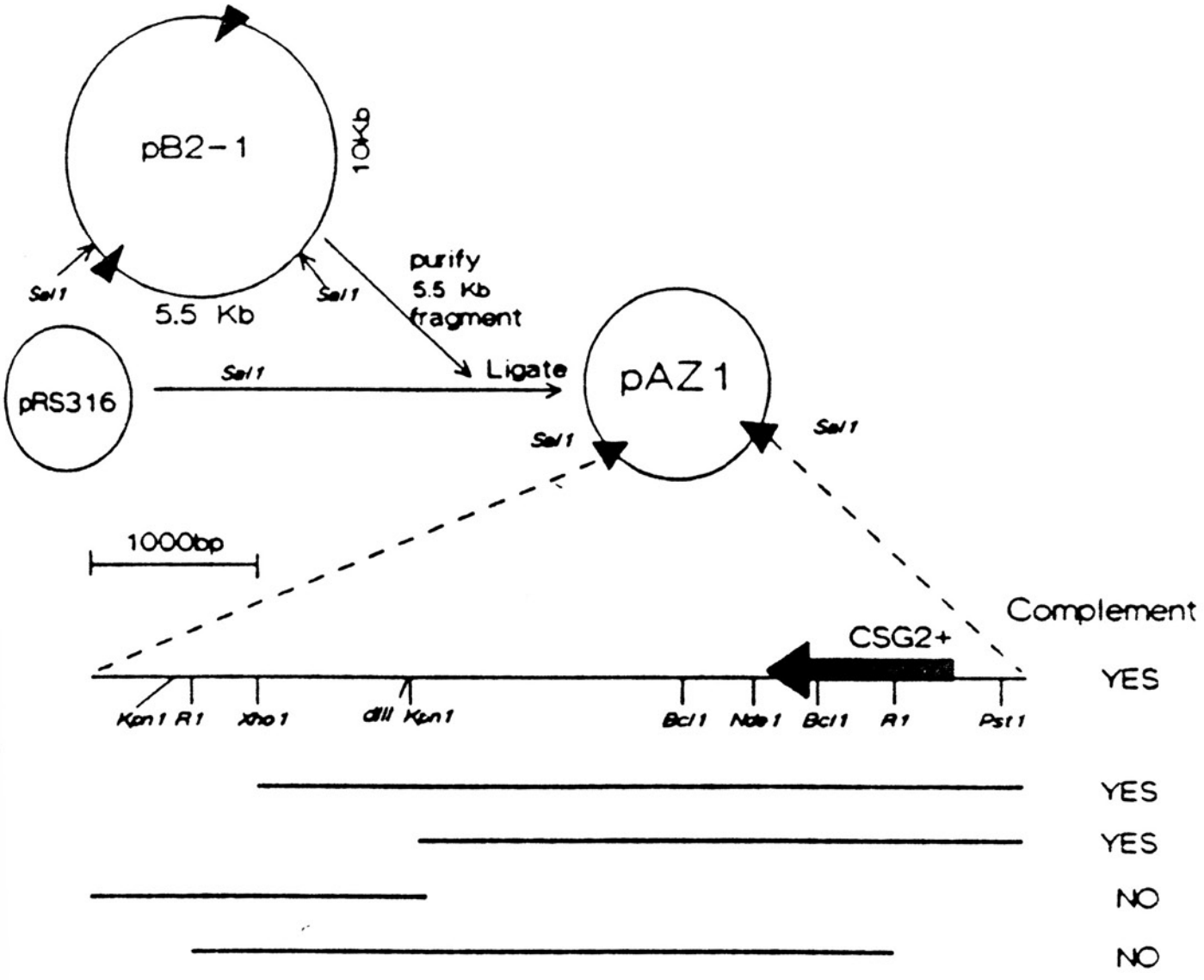
4. Bacterial strains *SCS1, AG1, XL1, JM109* (Stratagene) were used for the propagation of plasmids and M13 phage. Transformation of plasmid DNA into *E. coli.* competent cells was based on the protocol provided by "Stratagene".

5. Sequence determination was accomplished with the ABI 373A DNA Sequenator using the primer sequencing kit of ABI (Applied Biosynthesis, Inc.) for the M13mp18 and mp19 templates. The entire sequence was determined for both strands.

Localizing the CSG2 gene: To localize the *CSG2* gene on the complementing plasmid, fragments of the cloned yeast DNA were subcloned. A single *SalI* site within the insert separates the cloned yeast DNA into 5.5- and 10-kilobase fragments; subcloning of the 5.5-kilobase fragment into PRS316 [Sikorski and Hieter, 1989] localized the *CSG2* gene to the smaller *SalI* fragment (5.5 kb) (Figure 2). The complementing activity was further delineated by subcloning restriction fragments into PRS316 and testing for their ability to confer growth on media containing high Ca^{2+} concentration.

Construction of the CSG2 null allele: One step gene replacement was used to disrupt the *CSG2* gene [Rothstein, 1983]. A null allele of *CSG2* in which about 800 bp of *CSG2* coding sequence was replaced with the $LEU2^{+}$ gene was generated by inserting the *KpnI* to *PstI* fragment carrying *CSG2* (Figure 2) into PRS306 [Sikorski and Hieter, 1989]; this plasmid was then linearized with *EcoRI*, incubated with *Bal31*

Figure 2. Localization of the *CSG2* gene on the complementing plasmid and restriction map of pAZ1.


pB2-1
10Kb
Sal I
5.5 Kb
Sal I
purify
5.5 Kb
fragment
pRS316
Sal I
Ligate
pAZ1
Sal I
Sal I
1000bp
Complement
CSG2+
YES
Kpn I
R1
Xho I
dIII
Kpn I
Bcl I
Nde I
Bcl I
R1
Pst I
YES
YES
NO
NO

to remove about 800 bps and a *XhoI* linker was ligated in at the deletion junction. A *SalI* fragment carrying the *LEU2*$^+$ gene was ligated into the *XhoI* site and the *KpnI* to *PstI* fragment of this plasmid was used to transform the diploid strain TDY821 to *LEU2*$^+$. *CSG2*$^+$*/csg2::LEU2*$^+$ heterozygotes were identified by Southern Blot analysis [Southern, 1975].

The expression of CSG2 protein: Total yeast RNA was extracted in the presence of guanidinium thiocyanate and purified by ultracentrifugation on cesium chloride gradients [Campbell and Duffus (a), 1988]. Northern blot analysis was done to determine the level of *CSG2* mRNA [Maniatis *et al.*, 1982].

Epitope tagging of CSG2 gene: An oligonucleotide primer was synthesized containing the last codon (CAT-his) and nine following deoxynucleic acids of the *CSG2* gene with the 12CA5 hemagglutinin epitope (TACCCATACGACGTCCCAGACTACGCT) [Guthrie and Fink, (b)1991] inserted in-frame between CAT and TAC. The epitope was tagged into *CSG2* gene by using oligonucleotide-directed *in vitro* mutagenesis system version 2 (Amersham International plc). The *Kpn1*$_2$*-Sal1* fragment containing *CSG2* gene was cloned into M13 vector. The synthesized oligonucleotide was annealed to the single-stranded DNA form of the vector and served as a primer for *in vitro* synthesis of the complementary DNA strand. The inserted epitope was detected by spotted Southern blot using the α-[^{32}P]dATP labeled epitope as the probe and proved by sequencing

analysis.

RESULTS:

1. The Ca^{2+} specific collection:

To identify Ca^{2+} sensitive mutants, 60,000 EMS(Sigma #M-0880)-mutagenized cells (approximately equal number of **a** and **α**-mating) were screened for the inability to grow at 37°C on YPD plates containing 100 mM Ca^{2+}. The mutants were divided into two classes according to whether they were sensitive only to Ca^{2+} or whether they were sensitive to both 50 mM Ca^{2+} and 50 mM Sr^{2+}. Secondary biochemical screens were performed on the Ca^{2+}-sensitive mutants to determine their cellular Ca^{2+} loading after incubation in YPD + 10 mM Ca^{2+} for 1.5 hours at 37°C, and the capacity for vacuolar Ca^{2+} transport by digitonin-permeabilized cells in the presence of 27 μM Ca^{2+} and 1 mM ATP.

Eighteen of the Ca^{2+}-sensitive mutants out of the total 64 were able to grow in the presence of 50 mM Sr^{2+}. Complementation analysis of these *csg* (Calcium Sensitive Growth) mutants identified two major complementation groups (*csg1* and *csg2*) and eight independent isolates (Table I).

Among the Ca^{2+}-specific mutants, those in the *csg1* and *csg2* complementation groups had an average 315% and 1090% increase in the cellular Ca^{2+} relative to wild type when incubated for 1.5 hours in YPD + 10 mM Ca^{2+} at 37°C. All other Ca^{2+}-specific mutant isolates showed wild type levels of Ca^{2+} accumulation except for the mutant strain TDY 2022 which showed a 680% increased Ca^{2+} accumulation [Beeler

Table I. Complementation analysis of *CSG* mutants. Diploids were tested for growth on 100 mM Ca^{2+} at 37°C. Failure of the diploid to grow (-) indicates that both haploids have a mutation in the same gene.

		csg1				*csg2*					
		2000	**2001**	**2002**	**2036**	**2003**	**2041**	**2020**	**2018**	**2022**	2042
	2005	−	−	−	−	+	+	+	+	+	+
csg1	2006	−	−	−	−	+	+	+	+	+	+
	2031	−	−	−	−	+	+	+	+	+	+
	2035	+	+	+	+	−	−	+	+	+	+
csg2	2010b	+	+	+	+	−	−	+	+	+	+
	2004	+	+	+	+	+	+	+	+	+	+
	2008	+	+	+	+	+	+	+	+	+	+
	2026	+	+	+	+	+	+	+	+	+	+
	2032	+	+	+	+	+	+	+	+	+	+

et al., 1994].

2. The *csg2* mutation is a recessive single nuclear mutation:

Growth of a heterozygous *csg2-1/CSG2*$^+$ diploid was normal on YPD + 100 mM Ca^{2+}, indicating that the *csg2* mutation is recessive. Tetrad analysis of the heterozygous diploid showed 2 Ca^{2+}-sensitive : 2 Ca^{2+}-resistant spore colonies for each 4-spored tetrad, indicating that the *csg2* phenotype is due to a single nuclear mutation.

3. *CSG2* gene was cloned by complementing the Ca^{2+}-sensitive growth of *csg2-1* cells:

The wild type *CSG2* gene was cloned by selecting for its ability to complement the *csg2-1* Ca^{2+}-sensitive phenotype [Beeler *et al.*, 1994]. The *csg2* cells were transformed with a YCp50-based genomic library [Rose *et al.*,1987]. The complementing plasmid, that conferred Ca^{2+}-resistance to the mutant cells, was selected by replica plating the *URA3*$^+$ transformants to YPD + 100 mM Ca^{2+} plates. When the Ca^{2+}-resistant transformants were grown on YPD to allow plasmid segregation, the resulting *ura*$^-$ segregants (selected on 5-fluoroorotic acid plates [Boeke *et al.*, 1984]) simultaneously reverted back to the Ca^{2+}-sensitive phenotype. These results indicated that the plasmid, pB2-1, contained an insert common to all the complementing plasmids (Figure 2).

To demonstrate linkage of the cloned DNA to the *CSG2* locus, we constructed

an integrating plasmid by subcloning a 2000-base pair *HindIII-SalI* fragment from the yeast DNA insert of pB2-1 in YIp5. The resultant plasmid was used to integratively transform a wild-type (DBY947) strain thereby marking the chromosomal locus homologous to the insert of pB2-1 with the URA^+ gene. A URA^+ transformant having the YIp5 plasmid integrated at the appropriate locus (confirmed by Southern blot analysis [Southern, 1975]) was mated to a *csg2-1* haploid, the resulting diploid was sporulated, and tetrads were dissected. In all 16 tetrads analyzed, parental ditype segregation of the Ca^{2+}-sensitive and URA^+ phenotype was observed, indicating that the cloned fragment included the wild-type *CSG2* gene.

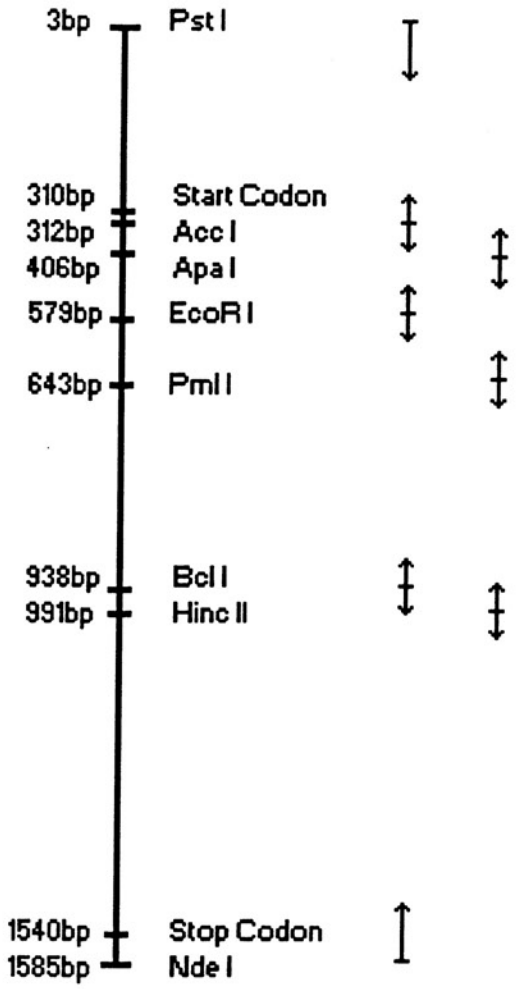
The *CSG2* gene was localized within the complementing fragment of genomic DNA by subcloning various restriction fragments and testing their ability to complement the *csg2-1* Ca^{2+}-sensitive phenotype. (Figure 2) The *XbaI-PstI* and $KpnI_2$-*PstI* fragments complemented the *csg2* Ca^{2+}-sensitive phenotype, but the $KpnI_1$-$KpnI_2$ and $EcoRI_1$-$EcoRI_2$ fragments did not, indicating that the *CSG2* gene resides within the $KpnI_2$-*PstI* fragment and that the $EcoRI_2$ site is inside the gene.

4. *CSG2* gene sequence:

The *CSG2* gene was sequenced by use of the primer sequencing kit of ABI with M13mp18 and M13mp19 subclones used as templates. Figure 3 shows a partial restriction map of the *CSG2* gene including enzymes which were used for constructing the subclones; positions of the primers used for sequencing are indicated. For some sequencing subclones, a set of *Bal31* deletions generated from

Figure 3. The strategy for sequence determination of the *CSG2* gene. Arrows represent the starting sites and directions of sequence reactions.

CSG2 Map


3bp
Pst I
310bp
Start Codon
312bp
Acc I
406bp
Apa I
579bp
EcoR I
643bp
Pml I
938bp
Bcl I
991bp
Hinc II
1540bp
Stop Codon
1585bp
Nde I

the *EcoRI* site were used.

The entire nucleotide and deduced amino acid sequence of the predicted 45,579-dalton protein is presented in Figure 4. A comparison of the *CSG2* open reading frame (ORF) to sequences in the NBRF and EMBL GenBank databases showed that an amino-terminal portion of the *CSG2* ORF is identical to an unidentified ORF at the end of the cloned 1.7 kb DNA fragment that also contained the full length *SCO1* gene. Since the *SCO1* gene was mapped to chromosome II, *CSG2* is located on chromosome II also [Schulze and Rodel, 1989; Schulze and Rodel, 1988].

The hydropathy analysis of *CSG2* indicated the existence of up to nine transmembrane sequences (Figure 5A). A possible topological representation of CSG2p is shown in Figure 5B. A potential signal sequence was found at the amino terminus. This region contains a sequence of 12 hydrophobic amino acids followed by two potential signal peptide cleavage sites (TKC->LS->N) [von Heijine, 1983a; von Heijine, 1983b]. Four consensus N-linked glycosylation sites were found; however, two of the four are placed on the cytoplasmic side of the membrane in our model, suggesting that only two of the sites are available for glycosylation. A 12-amino-acid segment with homology to a consensus Ca^{2+}-binding loop of the EF-hand type was found in the first cytoplasmic loop (Figure 5B) and is underlined in Fig. 4. The comparison of this site to the consensus Ca^{2+}-binding site as determined by Marsden *et al.* [da Silva *et al.*, 1991; Marsden *et al.*, 1990] is shown in Figure 6.

Figure 4. Nucleotide and translated amino acid sequence of *CSG2*. The sequence of a putative Ca^{2+}-binding site is underlined.

```
-309                                                                GCCTGCAGA
-300  GCAGCGCGCCAAGCAGAAGGAGGCATGGTACTCCTTCTTATTCAAATAAGATTGTAAATATATGCTACTTTCTTC
-225  CTTGTATATACATATATACTTATAACGCTATAACCCGTTCTTGTAATATCGGCTATCACCCGGCGAAGGTGTATC
-150  GCAAGAAAAAAAAAAAGCTCTCTCACTCTAAAGGAGGCTATGTGAACGTGGGAAGATCAAGTGAAAGAAATGTTTG
-75   TAGGCCATTTTCTTCCAGAACAGATCCGCTCTTGAGCTGGTGAGTTAGCACGATAACAAACAAAGATACAGCGTC

+1   ATGTCTACCACACTACTTTGGTTTTCAAGTGTAATAGGCTACGTGATTCAAACAAAATGTTTGTCTAACATACAA
       M  S  T  T  L  L  W  F  S  S  V  I  G  Y  V  I  Q  T  K  C  L  S  N  I  Q   +25

+76  TCTAAAAAGGAAATCTCCGTGGGGCCCAATGGTACAATTGCAACGCCTGAAACTAACGGCGACAACGGAAACTCA
       S  K  K  E  I  S  V  G  P  N  G  T  I  A  T  P  E  T  N  G  D  N  G  N  S   +50

+101 AGTTCATTAACCTTCTATCTGACCTTTATGTATTTTGCTTCGTGGCTGCTCTGGGTGCCTGCATCTCGACTTTGG
       S  S  L  T  F  Y  L  T  F  M  Y  F  A  S  W  L  L  W  V  P  A  S  R  L  W   +75

+176 GAGAAGATGAGACCGATGTTTGTCTCTGACTCAGACTCGAACAGGAATTCTCAGTTTGACAACAACAACAGCGGG
       E  K  M  R  P  M  F  V  S  D  S  D  S  N  R  N  S  Q  F  D  N  N  N  S  G   +100

+251 TCTGTGACAAACGAAGATGTCGATACGTTCTCGCACGTGTTGGATGATCCTCAACCACGGATTCCAGCCCAACAG
       S  V  T  N  E  D  V  D  T  F  S  H  V  L  D  D  P  Q  P  R  I  P  A  Q  Q   +125

+326 CAGAAGCAAAAAATCATATCCGTGGCTACCTTCAAATATGTGGCTAAGCTAACAGTGCTGGCTCTCATAATGATT
       Q  K  Q  K  I  I  S  V  A  T  F  K  Y  V  A  K  L  T  V  L  A  L  I  M  I   +150

+401 GTCGCTGATTTGACTTATAACATGGCTTTGTCATTGTCACCGGCATTTGATGTTGCTTTGATGCAAAATACTGCC
       V  A  D  L  T  Y  N  M  A  L  S  L  S  P  A  F  D  V  A  L  M  Q  N  T  A   +175

+476 ATTTTCGAAATTGTCACTTTACTATATGGTGTTTGTGGAATCTCCAGGAAGAACTACGTTTTCCGTAATTTCCTC
       I  F  E  I  V  T  L  L  Y  G  V  C  G  I  S  R  K  N  Y  V  F  R  N  F  L   +200

+551 ATCATGATGAACGCGGTCATTGGAATTTTGATCATCTCATACACGAAGGCTACCTGTGACATGCTTGCCGGAAAG
       I  M  M  N  A  V  I  G  I  L  I  I  S  Y  T  K  A  T  C  D  M  L  A  G  K   +225

+626 CTGTCCGTCAACCCTAACACGGGTGAACTTTCTGACCCATTCTTGTTTGATAGGTTGAAAGGTGCTCTGATTTGC
       L  S  V  N  P  N  T  G  E  L  S  D  P  F  L  F  D  R  L  K  G  A  L  I  C   +250

+701 GGCCTTGGTGCTTTGATTATGGGTCCTTTTGCCGTGTTATGGAACCGTTGGTTTTGCAGTAACATTTCCAAGAAC
       G  L  G  A  L  I  M  G  P  F  A  V  L  W  N  R  W  F  C  S  N  I  S  K  N   +275

+776 GAAAATTCTGCTGTAGTCTTGGTTAAGCAGAGCACCCACATGGCCCTAATCGGTATTATTGGCATGGTAATACTT
       E  N  S  A  V  V  L  V  K  Q  S  T  H  M  A  L  I  G  I  I  G  M  V  I  L   +300

+851 TTGCCATTTATTCCTAAATTTCCCTCCCGTGAGTCTGTGGAATCCATTTCGTTGTTCTATAATGACAAGAGCTTT
       L  P  F  I  P  K  F  P  S  R  E  S  V  E  S  I  S  L  F  Y  N  D  K  S  F   +325

+926 TGGTTCTCTCTACTAGGCTCGATTATCTTTGGTTCCTTGCCGAGCTTGATTTCGATATTAGAGTTGAATCGCAAG
       W  F  S  L  L  G  S  I  I  F  G  S  L  P  S  L  I  S  I  L  E  L  N  R  K   +350

+1001 GCCCCTGCTGAGTATTTGACGACGTGCAACCTGGGAGCTATTATCTTTATGGGGTTAGCTGAGTGGGTTTGCGAA
       A  P  A  E  Y  L  T  T  C  N  L  G  A  I  I  F  M  G  L  A  E  W  V  C  E   +375

+1076 CCTACGCAAACCACACAATTGTGAGATGGGAAGTCATAGGATACATAATGCTAACGGTAAGTTTGTTGGTCCTATCA
       P  T  Q  T  T  I  V  R  W  E  V  I  G  Y  I  M  L  T  V  S  L  L  V  L  S   +400

+1151 GTAACACTCGGGGAAGGTAAATACCACCATTAGTACGGACAACATACTTTATATGACTGATGATGATGTAACAAG
       V  T  L  G  E  G  K  Y  H  H  U                                              +410

+1226 CATATGCAATTATTTGGTTTCCCTAATAAAAAATCCCAATCAATATCTTGATTATTTTCAAGACAATTACTAGG
+1301 ATGTTCTTCTATTTTTTTATTTTTATTTGTATATTGTATATTCTAAAAAGAAGAAGCCATTTGGTGGGCTTTATT
+1376 ATAAATATTAAGAGGCATACCTCCGCCTATCCGCCTATCCTCGTCATGAACAATCAATTAAAAGCGCTTATAAAG
+1451 CGATATACAAG
```

Figure 5. Identification of membrane spanning segments and potential glycosylation and Ca^{2+}-binding site on CSG2p. Panel A, Kyte and Doolittle [1982] hydropathy plot of CSG2p. Each point on the curve represents a hydropathy average of a 14-amino-acid window centered at that point. Proposed transmembrane domains are numbered 1-9. Panel B, schematic representation of CSG2p showing possible signal peptidase cleavage sites, a putative Ca^{2+}-binding site, 2 N-linked glycosylation sites, and transmembrane segments.

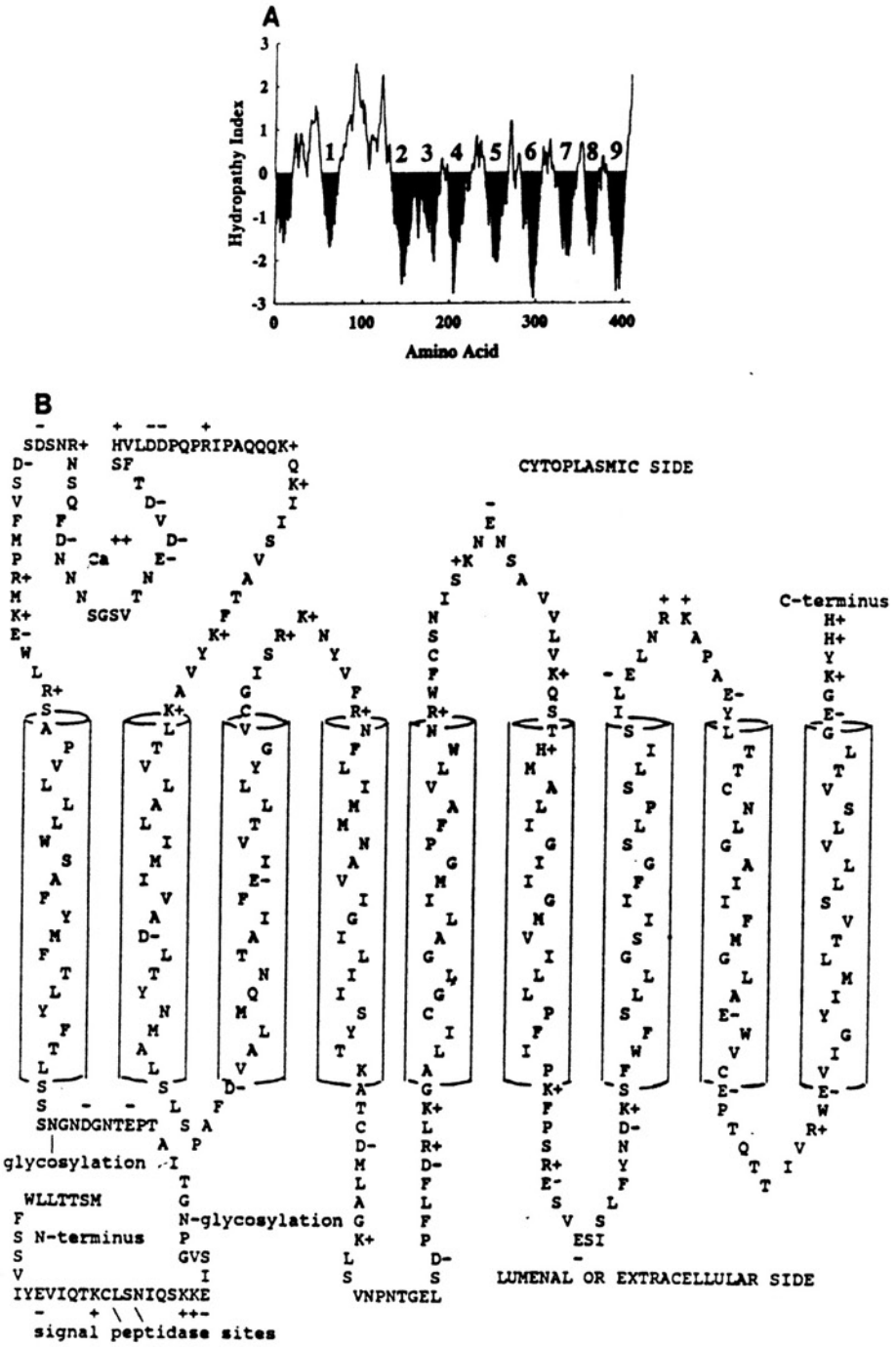

A
Hydropathy Index
Amino Acid
1
2
3
4
5
6
7
8
9
B
CYTOPLASMIC SIDE
C-terminus
LUMENAL OR EXTRACELLULAR SIDE
glycosylation
N-glycosylation
N-terminus
signal peptidase sites

Figure 6. Comparison of the amino acid sequence of a putative Ca^{2+}-binding site (see Fig.4) and the consensus Ca^{2+}-binding site of the EF hand. The amino acids typed in bold are the ones found in CSG2p.

CSG2 Sequence 92-103											
Asp	Asn	Asn	Asn	Ser	Gly	Ser	Val	Thr	Asn	Glu	Asp
Amino acids that occur in the Ca^{2+}-binding loop of EF-hands with the percentage of total amino acids found											
Position in Ca^{2+}-Binding Loop											
1	2	3	4	5	6	7	8	9	10	11	12
ASP	Lys	Asp	Gly	Asp	**GLY**	Phe	Ile	Asp	Phe	**GLU**	Glu
98	28	73	51	56	89	16	70	33	16	31	86
Asn	Ala	**ASN**	Lys	**SER**	Asp	Tyr	**VAL**	Ser	Val	Asp	**ASP**
0.6	10	24	14	22	2	15	13	19	12	27	9
Tyr	Thr	Glu	**ASN**	Asn	Asn	Lys	Leu	Glu	Ala	Ala	Val
0.6	10	2.5	9	17	2	13	9	14	10	10	2
Glu	Gln	Ser	Arg	Gly	Lys	Thr	Met	**THR**	Glu	Lys	Asn
0.6	9	0.6	6	2	2	13	2.5	11	10	8	1
–	Val	–	Ala	Gln	Arg	Glu	Cys	Gly	Leu	Gln	Gln
–	8	–	5	0.6	1	6	2	10	10	5	0.6

5. Biochemical phenotypes of the *csg2* null mutant:

The *csg2* null allele was constructed under Material and Methods by substituting most of the *CSG2* coding sequence with a *LEU2*$^+$ marker. The *csg2* null mutant cells were viable and grew comparably to wild type on YPD medium but failed to grow on the same medium containing 50 mM Ca^{2+} [Beeler *et al.*, 1994]. The null allele was similar to the EMS-derived *csg2* alleles with regard to growth properties and biochemical phenotypes. Hence it is the lack of functional CSG2p that causes Ca^{2+}-sensitivity. The Ca^{2+} transport properties of the *csg2* null mutant were the same as those of the EMS-induced *csg2* mutants.

The rate as well as the amount of Ca^{2+} accumulated by *csg2Δ* cells in 10 mM Ca^{2+} was greater than that of wild type. Sr^{2+} accumulation by *csg2Δ* cells was increased 2.5 times while Ca^{2+} accumulation increased 22 times indicating that Ca^{2+} accumulation was more influenced by the *csg2* mutation. In wild-type cells, most cellular Ca^{2+} is localized in the vacuole where it forms a stable, nonexchangeable complex with polyphosphate [Dunn *et al.*, 1994]. The *csg2* mutant had normal vacuolar Ca^{2+} accumulation which was inhibited by Mg^{2+}, but increased accumulation into a non-vacuolar organelle which was not influenced by Mg^{2+}.

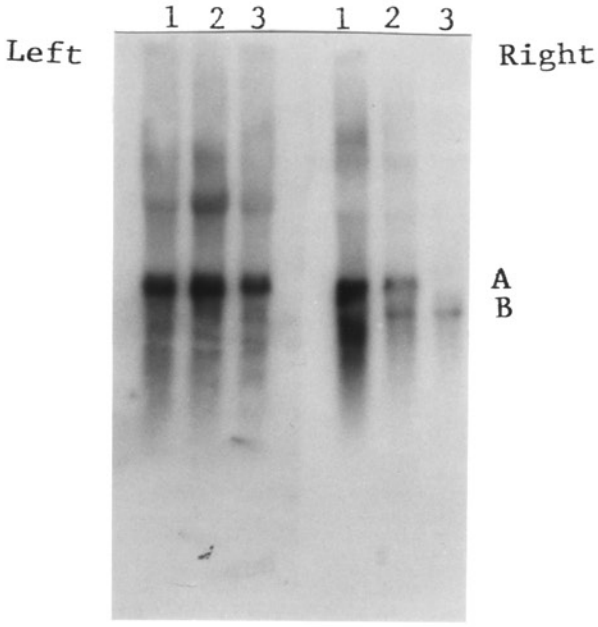
6. The expression of CSG2p is not induced by Ca^{2+}:

Because the *csg2Δ* cells displayed a Ca^{2+}-sensitive phenotype, CSG2p is required for growth in high Ca^{2+}-containing media. Thus we addressed whether *CSG2* gene expression varies with Ca^{2+} in the media. The level of *CSG2* mRNA in

cells grown in high Ca^{2+} (100 mM) medium was compared with that in cells grown in low Ca^{2+} (3.0 mM) medium by Northern blot analysis [Maniatis *et al.*, 1982].

Total RNA was prepared by the protocol described in the Material & Methods section. Twenty micrograms of RNA were loaded per lane. An actin probe was used as a control for loading. Because the *CSG2* mRNA was hard to detect, the *CSG2* gene was cloned into a high copy vector (Yep24, 2 μm plasmid) to generate the plasmid pAZ2 which was transformed into wild-type cells. In figure 7, RNA from wild-type cells (containing plasmid pAZ2) cultured with YPD (containing 0.3 mM Ca^{2+}) (lane 1) and YPD + 100 mM Ca^{2+} (lane 2) was compared to RNA from *csg2Δ* mutant cells (lane 3). The left panel was probed with a labeled oligonucleotide of actin gene which was used as the loading control. The probe used in the right panel was a labeled oligonucleotide containing the C-terminal fragment of *CSG2* gene (Band A) as well as part of the downstream gene (Band B). In the Right panel, band A is missing in lane 3 which is the *csg2Δ* strain. It can be seemed that lane 1 was overloaded by comparing the amount of the downstream gene as an internal control. The expression of *CSG2* and the downstream gene were at the same level relative to one another in both lane 1 and lane 2, indicating that increasing the Ca^{2+} concentration in the media has no influence on the expression of the *CSG2* gene. Because the same amount of RNA was loaded in each lane in Fig. 7, by comparing the signal in lane 2 of panel A to that of panel B, the level of *CSG2* transcript is much lower than that of the actin mRNA suggesting that csg2p may be a low-abundance protein.

Figure 7. Northern blot analysis of the expression of the *CSG2* gene. Panel Left: The oligonucleotide probe was from actin gene. Actin was used as a control for highly expressed protein. Panel Right: The oligonucleotide probe was a *HincII* fragment that covered the C-terminus of *CSG2* gene and a part of its downstream gene. Lane 1, 2, and 3 were loaded with RNA from wild-type cultured with YPD, wild-type cultured with YPD + 100 Mm Ca^{2+}, and *csg2Δ* strains respectively. The wild-type strain were transformed with a high copy plasmid (Yep 24) containing *CSG2* gene. Band A and B represent *CSG2* gene and the downstream gene respectively.


1 2 3 1 2 3
Left
Right
A
B

7. Attempts to construct trpE fusions:

Fragments of the *CSG2* gene were fused in-frame to the *E. coli trpE* gene in the pATH vectors [Guthrie and Fink (a), 1991]. None of the fragments gave rise to a stable fusion protein in *E. coli*, suggesting that the *CSG2* protein may be toxic to *E. coli* cells. The *CSG2* gene was epitope-tagged with an hemagglutinin epitope [Guthrie and Fink (b), 1991] at the carboxyl terminus. The tagged allele complemented the *csg2Δ* mutant, suggesting that the *CSG2* was expressed and localized properly. However, no band was detected in Western blot analysis using the 12CA5 antibody.

CHAPTER THREE

Suppressors of the *csg2* Ca^{2+} Sensitive Mutant

INTRODUCTION:

The deduced amino acid sequence of *CSG2* gene indicates that this gene encodes a novel membrane protein of unknown function. Attempts to construct CSG2p-trpE fusion protein for generation of antibodies were unsuccessful as well as the epitope-tagging strategy. Because CSG2p is not abundant, it is difficult to analyze it biochemically. Also, CSG2p shows no extensive homology apparently to any proteins in the protein sequence database. To gain more information about *CSG2*, we undertook a suppressor analysis of the *csg2* null mutant. Through suppressor analysis, genes that can mutate to circumvent the Ca^{2+}-sensitive defect caused by the mutation in the *CSG2* gene can be identified. Because suppressors are selected in the presence of the *csg2* null allele, all of them will be bypass suppressors which is defined as mutations occur in other genes to circumvent the effects of mutation in the original gene.

Suppressors of *csg2Δ* might identify genes that encode proteins that mediate Ca^{2+} influx into the overaccumulating organelle, that prevent interaction with Ca^{2+} required to cause cell death, that mediate the release of Ca^{2+} to block Ca^{2+} overaccumulation, or eliminate the synthesis of the molecule (such as polyphosphate) that precipitates the overaccumulated Ca^{2+} *etc.*

MATERIAL & METHODS:

Strains and *Media* were as described in Chapter Two.

Chemicals: Arsenazo III was obtained from Aldrich. Zymolyase 100T was supplied by Seikagaku Kogyo, Rockville, MD. All other chemicals were purchased from Sigma.

Whole cell Ca^{2+} accumulation: To identify mutants in the suppressor collection likely to have a defective Ca^{2+} transport system, the amount of Ca^{2+} accumulated by cells grown in YPD + 100 mM $CaCl_2$ was determined. The cells were harvested and washed by centrifugation and the amount of Ca^{2+} stored in the cells was determined spectrophotometrically using the Ca^{2+} indicator arsenazo III as previously described by Dunn *et al.* [1994].

ATP-dependent Ca^{2+}-uptake in vacuole: Ca^{2+} accumulation by intact vacuoles was measured by using osmotically shocked, partially regenerated spheroplasts which were prepared as described by Groesch *et al.* [1992]. The protein concentration was determined by use of the dye-binding method of Braford [Braford, 1976]. Two ml of 0.1 M KCl, 10 mM K MES, 10 mM K PIPES, 10 mM K HEPES (pH 7.0), 2 mM $MgSO_4$, 100 μM $CaCl_2$ and 50 μM arsenazo III containing spheroplasts of 1 mg protein was placed in a cuvette at 27°C. The amount of Ca^{2+} absorbed or released from the cell was determined spectrophotometrically using an SLM-Aminco DW2c

dual wavelength spectrophotometer by measuring the changes of absorbance caused by the formation of the arsenazo III-Ca^{2+} complex at 660 nm using 685 nm as a reference wavelength. One mM ATP was added to stimulate the Ca^{2+} absorbance by the vacuole vesicles. A23187 (5 μM), which is a Ca^{2+} ionophore, was added to release the Ca^{2+} uptake by the vacuole vesicles.

RESULTS:

1. Suppressor analysis of *csg2*:

Earlier results have indicated that CSG2p is a membrane protein that is essential for growth in high Ca^{2+}-containing medium [Beeler *et al.*, 1994]. Second-site suppressors of the *csg2* null mutation were isolated in the hope of identifying other proteins that participate in conjunction with CSG2p to maintain Ca^{2+} homeostasis. Suppressors were isolated by streaking haploid *csg2Δ* cells from single colonies onto YPD + 100 mM Ca^{2+} plates at 37°C, and selecting one fast growing revertant per colony to obtain independent mutations. Suppressors of *csg2* null mutants can only be obtained by mutations in other genes that bypass the CSG2p requirement since most of the *CSG2* coding sequence was eliminated in this null allele. A total of 600 mutants of both mating types were isolated and 125 (25 of α strains and 100 of a strains) were picked for analysis. These mutants were named *scs* for Suppressors of Ca^{2+} Sensitivity. The rate of pseudoreversion was about 10^{-6}.

The suppressor mutants were mated with the *csg2* null mutant to determine if the suppressor mutations are recessive or dominant (Figure 8). As shown in figure

8, the resulting diploid was a homozygote for the *csg2Δ/csg2Δ* gene but a heterozygote for the *scs/SCS* gene. If the suppressor mutation is dominant, then the phenotype of the diploid would be Ca^{2+}-resistant. On the other hand, if the suppressor mutation is recessive, then the phenotype of the diploid would be Ca^{2+}-sensitive. Growth of all *scs/SCS csg2/csg2* diploids was inhibited by 100 mM Ca^{2+}, indicating that the *scs* mutations are recessive.

Complementation analysis was performed to determine how many genes can be mutated to give *csg2* suppressors. The *scs* strains of one mating type were mated with those of the other, and the resulting diploids were tested for growth in 100 mM Ca^{2+}. As displayed in Figure 9, when two suppressor mutations are in different genes (Figure 9A), the diploid is a heterozygote for each of them and the phenotype is Ca^{2+}-sensitive since the mutations are recessive. When two suppressor mutations are in the same gene (Figure 9B), the diploid is a homozygote for the *scs* gene and the phenotype is Ca^{2+}-resistant. Mutant cells that failed to complement were placed in the same complementation group; the suppressor mutants (84%) fell into 7 complementation groups (*scs1-scs7*) (Table II).

2. Secondary phenotypes of suppressor mutant strains:

Suppressors of the *csg2* phenotype (Ca^{2+}-induced death) that acquire the reverse phenotype(Ca^{2+}-required for growth) may be expected to identify genes required for Ca^{2+} homeostasis. For example, a suppressor mutant might act by preventing Ca^{2+} uptake by the *csg2* cells. Therefore, the *scs* mutant strains were

Figure 8. Strategy of testing whether a mutation is recessive or dominant by a genetic approach. The Ca^{2+}-sensitive phenotype of diploids was tested on YPD + 100 mM Ca^{2+} plates.

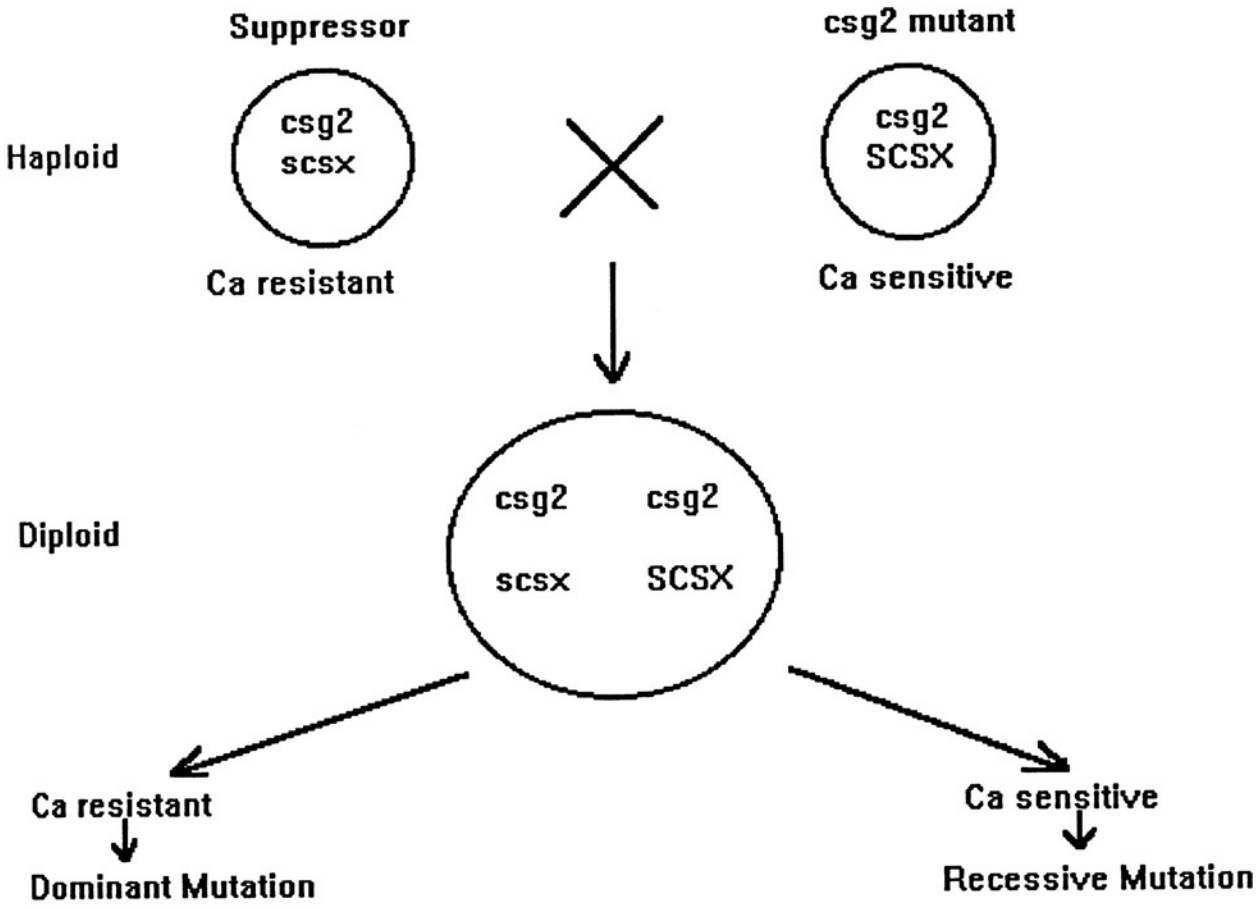
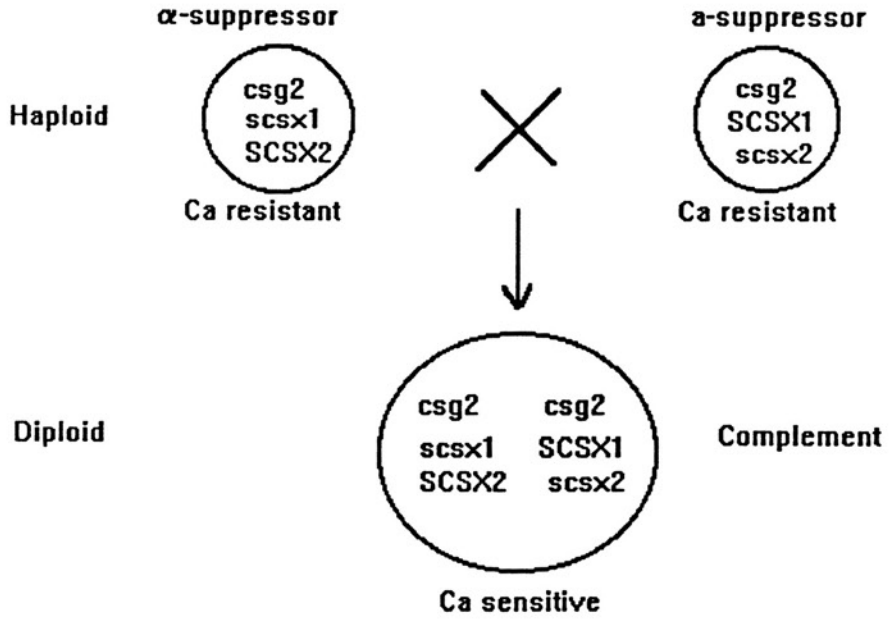

Suppressor
csg2 mutant
Haploid
csg2
scsx
csg2
SCSX
Ca resistant
Ca sensitive
Diploid
csg2
csg2
scsx
SCSX
Ca resistant
Dominant Mutation
Ca sensitive
Recessive Mutation

Figure 9. Strategy of complementation analysis. The Ca^{2+}-sensitive phenotype of diploids was tested on YPD + 100 mM Ca^{2+} plates.

A: Two mutations are in different genes:



B: Two mutations are in the same gene:

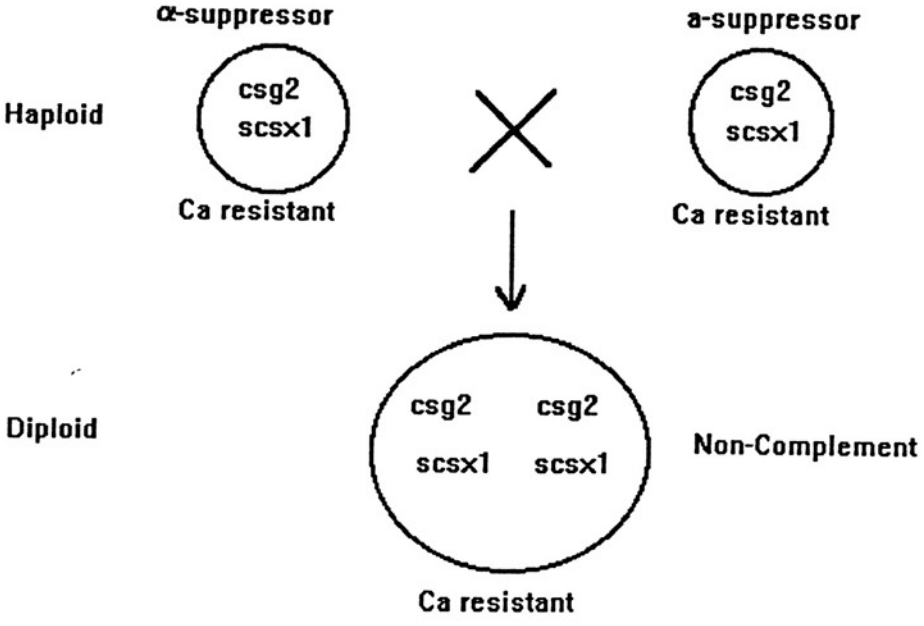
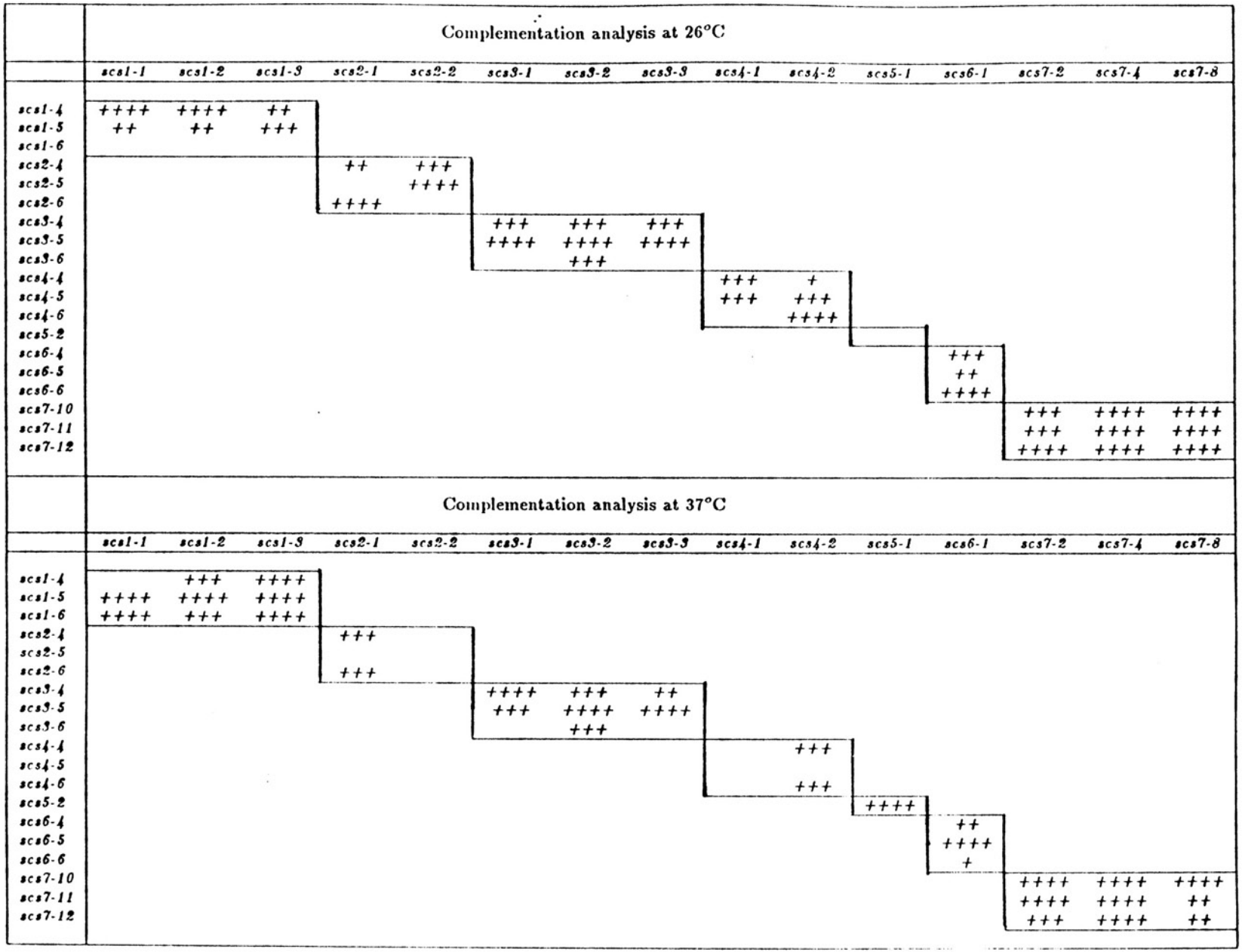


Table II. Complementation analysis of *scs* mutants. Haploid strains (vertical, Mat**a** *scsx csg2::LEU2*$^{\pm}$ *ade2-101 ura3-52 leu2Δ trp1Δ*; horizontal, Mat**α** *scsx csg2::LEU2*$^{\pm}$ *lys2 ura3-52 leu2Δ trp1Δ*) were mated, diploids were selected on SD + 100 mM Mg^{2+} + uracil + tryptophan, and tested for growth on YPD + 100 mM Ca^{2+} plates at 26 ° C (top panel) and 37 ° C (bottom panel). Growth was scored as + + + + (wild type level) to + (poor growth). A blank indicates no growth. Because the *scs* mutations are all recessive, the ability of the *csg2/csg2* diploids to grow in 100 mM Ca^{2+} indicates the *scs* mutations are noncomplementing. Twenty-five *Mat**α** scs* strains were crossed to 100 *Mat**a** scs* strains; 12% are in *scs1*, 8% in *scs2*, 12% in *scs3*, 8% in *scs4*, 4% in *scs5*, 4% in *scs6* and 36% in *scs7*. Sixteen percent did not fall into a complementation group. This figure includes a subset of the data.


Complementation analysis at 26°C
scs1-1 scs1-2 scs1-3 scs2-1 scs2-2 scs3-1 scs3-2 scs3-3 scs4-1 scs4-2 scs5-1 scs6-1 scs7-2 scs7-4 scs7-8
scs1-4 ++++ ++++ ++
scs1-5 ++ ++ +++
scs1-6
scs2-4 ++ +++
scs2-5 ++++
scs2-6 ++++
scs3-4 +++ +++ +++
scs3-5 ++++ ++++ ++++
scs3-6 +++
scs4-4 +++ +
scs4-5 +++ +++
scs4-6 ++++
scs5-2
scs6-4 +++
scs6-5 ++
scs6-6 ++++
scs7-10 +++ ++++ ++++
scs7-11 +++ ++++ ++++
scs7-12 ++++ ++++ ++++
Complementation analysis at 37°C
scs1-1 scs1-2 scs1-3 scs2-1 scs2-2 scs3-1 scs3-2 scs3-3 scs4-1 scs4-2 scs5-1 scs6-1 scs7-2 scs7-4 scs7-8
scs1-4 +++ ++++
scs1-5 ++++ ++++ ++++
scs1-6 ++++ +++ ++++
scs2-4 +++
scs2-5
scs2-6 +++
scs3-4 ++++ +++ ++
scs3-5 +++ ++++ ++++
scs3-6 +++
scs4-4 +++
scs4-5
scs4-6 +++
scs5-2 ++++
scs6-4 ++
scs6-5 ++++
scs6-6 +
scs7-10 ++++ ++++ ++++
scs7-11 ++++ ++++ ++
scs7-12 +++ ++++ ++

screened to identify those that require increased Ca^{2+} concentrations for growth (Table III). Wild type cells grow normally with only trace amounts of Ca^{2+} (in the presence of 10 mM EGTA). About 2% of the 600 suppressor isolates showed a Ca^{2+}-requiring phenotype; they failed to grow in YPD (about 0.3 mM Ca^{2+}) but grew in the presence of more than 10 mM Ca^{2+} on YPD plates. These suppressors were from three of the seven complementation groups. All members of *scs1* require at least 10 mM Ca^{2+} for growth in YPD. As showed in Figure 10, wild type cells grow well in YPD and YPD + 100 mM Ca^{2+} plates at both 26 °C and 37 °C; *csg2Δ* cells only grow in YPD plates, whereas the *scs1-1* mutant cells (with or without *CSG2*) grow only on YPD + 100 mM Ca^{2+} plates. Members of the *scs2* group grow poorly on YPD plates and not at all in YPD liquid unless supplemented with 10 mM Ca^{2+}. A few members of the *scs7* group needed 10 mM Ca^{2+} for growth. None of the divalent cations tested (Sr^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Zn^{2+}, or Mn^{2+}) could substitute for Ca^{2+} (Table IV). Some *scs1* alleles were semipermissive in media with 100 mM Mg^{2+}. Both the suppressing phenotype and Ca^{2+}-requiring phenotype of some *scs1* and *scs2* mutants were temperature sensitive. We also observed that *scs4* had an interesting phenotype at 37 °C in that it grows normally on YPD + 10 mM EGTA plate, fail to grow in 0.3-10 mM Ca^{2+}, but grows in Ca^{2+} above 25 mM. As the Ca^{2+} concentration is increased, it showed a Ca^{2+} requirement for growth (Table III).

Since *csg2* mutants have increased cellular Ca^{2+} when incubated in high level of Ca^{2+}, Ca^{2+} accumulation by *csg2* strains containing the suppressor mutations was measured to determine whether the Ca^{2+} overaccumulation phenotype was reversed

Table III. Effect of Ca^{2+} on the Growth of *scs* Mutant Strains.

Cells of the designated strain were resuspended in 100 μl 20% glucose in 96-well plates. A metal-pronged replicator was used to transfer cells to YPD plates supplemented with either 10 mM EGTA or the indicated Ca^{2+}. Plates were incubated at 26°C (top) or 37°C (bottom) for 2 days. Level of growth was scored from "+++" (wild type level) to "+" (poor growth). No growth is indicated as "-".

Strain	10 mM EGTA	Ca^{2+} Concentration added to YPD (mM)					
		0	3	10	25	50	100
		Measured at 26°C					
wildtype	+++	+++	+++	+++	+++	+++	+++
csg2	+++	+++	+++	+/-	—	—	—
scs1-1	—	—	+	+++	+++	+++	+++
scs2-2	—	++	+++	+++	+++	+++	+++
scs3-2	+++	+++	+++	+++	+++	+++	+++
scs4-1	+++	+++	+++	+++	+++	+++	+++
scs5-1	+++	+++	+++	+++	+++	+++	+++
scs6-1	+++	+++	+++	+++	+++	+++	+++
scs7-8	+++	+++	+++	+++	+++	+++	+++
		Measured at 37°C					
wildtype	+++	+++	+++	+++	+++	+++	+++
csg2	+++	+++	+/-	+/-	—	—	—
scs1-1	—	—	+/-	+++	+++	+++	+++
scs2-2	—	++	+++	+++	+++	+++	+++
scs3-2	+++	+++	+++	+++	++	+	+
scs4-1	+++	+/-	+/-	—	++	++	+++
scs5-1	+++	+++	+++	+++	+++	+++	+++
scs6-1	+++	+++	+++	+++	+++	+++	+++
scs7-8	+++	+++	+++	+++	+++	+++	+++

Figure 10. The Ca^{2+}-requiring phenotype of *scs1-1*. Cells were streaked onto the indicated plates and incubated at either 26°C or 37°C for two days.

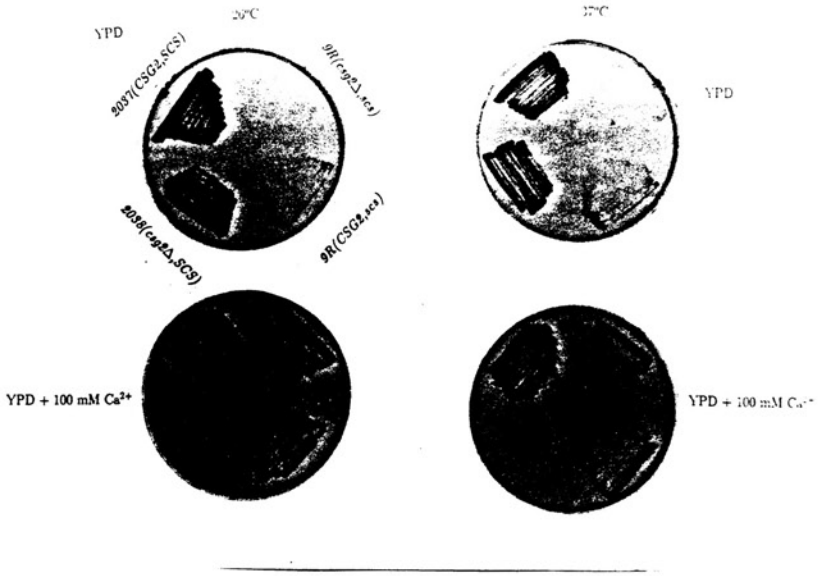

YPD
20°C
37°C
2037(CSG2,SCS)
9R(csg2Δ,scs)
2038(csg2Δ,SCS)
9R(CSG2,scs)
YPD
YPD + 100 mM Ca2+
YPD + 100 mM Ca2+

Table IV. Effects of divalent cations on the growth of *scs* mutant strains. Cells of the designated strain were resuspended in 100 μl 20% glucose in 96-well plates. A metal-pronged replicator was used to transfer cells to YPD and YPD supplemented with divalent cations at the indicated concentrations. Plates were incubated at 26°C for two days. Level of the growth was scored from "+ + + +" (wild-type level) to "+/-" (poor growth). No growth is indicated as "-". "pap" stands for "papilla".

Effects of Divalent Cations

26C	2037	2038	scs1-1	scs1-2	scs1-3	scs2-2	scs3-2	scs4-1	scs5-1	scs6-1	scs7-8
YPD+10mM EGTA	++++	++++	-	-	++++	-	++++	++++	++++	++++	-
YPD	++++	++++	-	-	++++	++++	++++	++++	++++	++++	++++
YPD+3mM Ca	++++	++++	+	++++	++++	++++	++++	++++	++++	++++	++++
YPD+10mM Ca	++++	-/+	++++	++++	++++	++++	++++	++++	++++	++++	++++
YPD+25mM Ca	++++	-	++++	++++	++++	++++	++++	++++	++++	++++	++++
YPD+50mM Ca	++++	-	++++	++++	++++	++++	++++	++++	++++	++++	++++
YPD+100mM Ca	++++	-	++++	++++	++++	++++	++++	++++	++++	++++	++++
YPD+10mM Mg	++++	++++	-	+	++++	++++	++++	++++	++++	++++	++++
YPD+25mM Mg	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
YPD+50mM Mg	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
YPD+100mM Mg	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
YPD+1mM Ni	++++	++++	-	-	++++	-	++++	++++	++++	++++	++++
YPD+3mM Ni	-	-	-	-	-/+	-	-	-	-/+	-	-
YPD+1mM Zn	++++	++++	-	+	++++	-	++++	++++	++++	++++	++++
YPD+3mM Zn	++++	++++	+	++	++++	-	++++	++++	++++	++++	++++
YPD+10mM Zn	-	-	-	-	+	-	-	-	-	-	-
YPD+1mM Co	-	-pap	-	-/+	++++	-	-pap	-	++++	++++	++++
YPD+3mM Co	-	-	-	-	-	-	-	-	-	-	-
YPD+1mM Mn	++++	++++	-	-	++++	+	++++	++++	++++	++++	++++
YPD+3mM Mn	++++	++++	-	+	++++	++++	++++	-/+	++++	++++	++++
YPD+10mM Mn	-	-	-	-	-	-	-	-	-	-	-
YPD+50mM Sr	++++	++++	++	+++	++++	++	++++	-	++++	++++	++++
YPD+50mM Ba	++++	++++	-	-	++++.	++	++++	+	++++	++++	++++

by the suppression mutation. Representatives from complementation groups (*scs1-scs7*) were chosen to be used in this measurement. After a 2 hour incubation at 26 °C in YPD + 100 mM Ca^{2+} , *csg2* mutant cells accumulated about 3 times the Ca^{2+} level found in wild type cells [Beeler *et al.*, 1994]. However *csg2* mutant cells containing the suppressor mutations (except *scs1*) accumulated wild type levels of Ca^{2+}. Although the *scs1-1 csg2* mutant continued to grow in 100 mM Ca^{2+} it accumulated much higher Ca^{2+} levels (4-fold) than wild type (Figure 11).

In wild-type cells, the vacuole is the major Ca^{2+} storage pool in yeast. Osmotic lysis of yeast spheroplasts provides vacuole vesicles that can be assayed for Ca^{2+} uptake. When ATP is added to the medium, the vacuolar membrane vesicles actively pump protons into the vesicles via the vacuolar H^+-ATPase. The resulting proton gradient drives Ca^{2+} uptake by the $Ca^{2+}/2H^+$ exchanger. The decrease of Ca^{2+} in medium can be measured spectrophotometrically. The wild type and suppressor strains were grown in YPD + 100 mM Ca^{2+} (pH 4.7), but the *csg2Δ* strain was grown in YPD (pH 4.7) medium. All suppressors (except *scs1-1*) showed vacuolar Ca^{2+}-uptake comparable to that measured for the wild-type (Figure 11). The *scs1-1* mutant showed a higher vacuolar Ca^{2+}-uptake (5-fold). These data show that suppressors had normal or higher vacuolar Ca^{2+} uptakes.

Figure 11. Ca^{2+} accumulation.

1.Ca^{2+} uptake by whole cells: Ca^{2+} (100 mM) was added to cells growing in YPD medium at 26°C. To determine Ca^{2+} loading, cells (in log phase) were harvested by centrifugation and washed three times with 10 mM Tris (pH 8.0) at 4°C to remove extracellular Ca^{2+}. The cells were then diluted to a concentration of 10^7 cells/ml into a solution containing 0.1 M KCl, 1 mM $MgSO_4$, and 100 μM arsenazo III. Intracellular Ca^{2+} was released from the cells by addition of 1 mg/ml digitonin and the absorbance of arsenazo III-Ca^{2+} complex (difference absorbance 660 nm-685 nm) was measured as described under Materials & Methods.

2. ATP-dependent Ca^{2+} accumulation by lysed spheroplasts: partially regenerated spheroplasts (5 mg/ml) were prepared as described under "Material & Methods" and resuspended in 10 mM Tris (pH 8.0), 1 M sorbitol. A 40-fold dilution of the partially regenerated spheroplasts in 0.1 M KCl, 10 mM K PIPES, pH 7.0, 10 mM K MES, 10 mM K HEPES, pH 7.0, 2 mM $MgSO_4$, 100 μM $CaCl_2$, and 50 μM arsenazo III (27°C) causes an osmotic shock which permeabilizes the plasma membrane without causing lysis or irreversible damage to the internal membranes. Ca^{2+} uptake was monitored spectrophotometrically by measuring the decrease in the absorbance of the arsenazo III-Ca^{2+} complex at 660 nm using 685 nm as a reference wavelength. At the end of each measurement the accumulated Ca^{2+} was released by the addition of 5 μM A23187. The absorbance change was calibrated by titration of the arsenazo III with calcium atomic absorption standard solution (Sigma).

Strain	Ca^{2+} Uptake by Whole Cells	ATP-Dependent Ca^{2+} Accumultion by Lysed Spheroplasts
	(nmol $Ca^{2+}/10^7$ cells)	(nmol Ca^{2+}/mg protein)
wild type	15	9
csg2::LEU2	ND	13
scs1-1	57	50
scs2-1	18	5
scs3-2	13	8
scs4-1	15	6
scs5-1	19	6
scs6-1	7	22
scs7-8	22	12

CHAPTER FOUR

SCS1 Encodes the Serine Palmitoyltransferase; *scs* Mutant Strains as well as *csg2* Have an Altered Sphingolipid Metabolism

INTRODUCTION:

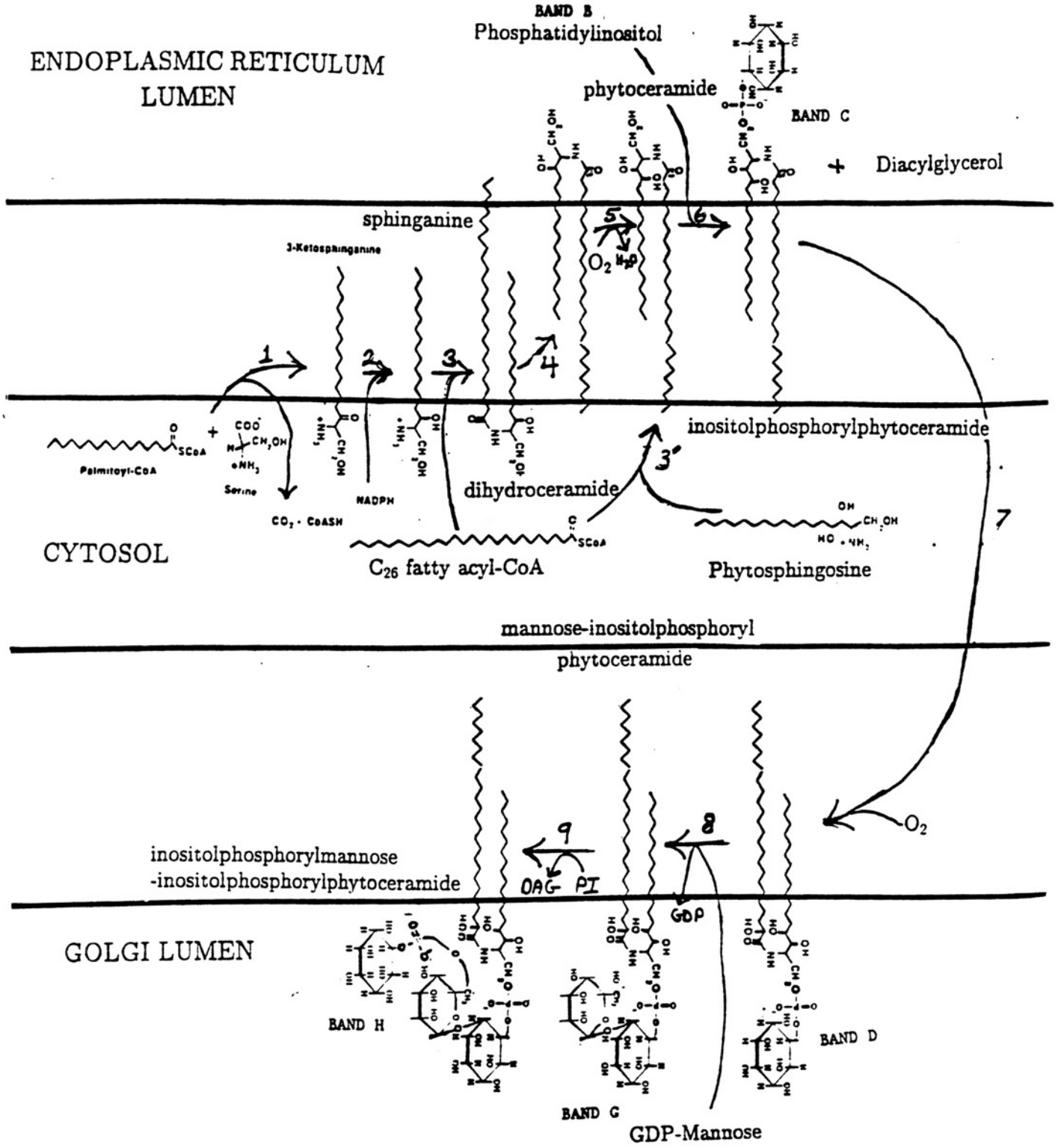
1. Sphingolipids in *S. cerevisiae*:

A. Sphingolipids are important components in the plasma membrane of yeast: In fact, sphingolipids are common and important components of all eukaryotic membranes. Sphingolipids were first discovered in human brain by Thudichum in 1884 [1884]. In mammalian cells, there are many types of sphingolipids, for instance, sphingomyelin, neutral sphingolipids (glucosylceramide, galactosylceramide, lactosylceramide, globoside etc), and acid sphingolipids (sulfatide, gangliosides). In the yeast *Saccharomyces cerevisiae*, there are three major sphingolipids: inositol-P-ceramide (IPC), mannose-inositol-P-ceramide (MIPC), and mannose-(inositol-P)$_2$-ceramide [M(IP)$_2$C]. They are composed of a hydrophobic segment (ceramide) and a polar head group. The ceramide consists of a long-chain base (sphingosine in animals, phytosphingosine (PHS) in fungi and plants) and a fatty acid in amide linkage. The polar head group in *S. cerevisiae* is different from that of animals in that it contains inositol, which is linked by a phosphodiester bond to the ceramide. The inositol is further substituted with polar groups such as mannose. The long chain bases in ceramide represent approximately 40% of the total inositol-containing lipids in *S. cerevisiae*. The intracellular distribution within different organelles of

sphingolipids is unequal. Patton & Lester [1991] found that 80 to 100% of the sphingolipids were localized in the plasma membrane. The sphingolipids constitute about 30% of the total phospholipid content of the plasma membrane.

B. Sphingolipid biosynthesis (Figure 12) [Merrill and Jones, 1990]: The initial precursors of *de novo* sphingolipid biosynthesis are palmitoyl-CoA and L-serine which are condensed by the action of serine palmitoyltransferase (EC 2.3.1.50), a pyridoxal 5'-phosphate-dependent enzyme. This enzyme has a high substrate specificity for palmitoyl-CoA; the best fit substrates are linear, saturated fatty acyl-CoA's of 16 ± 1 carbon atoms, which corresponds to the prevalence of 18-carbon sphingosine bases (C-3 to C-18 carbons from palmitic acid and C-1 and C-2 from serine). The cleavage of the reactive, high-energy, thioester bond of palmitoyl-CoA and the release of CO_2 from serine provide the driving force for the reaction. The next step in sphingolipid biosynthesis involves the reduction of the carbonyl group in 3-ketodihydrosphingosine. This reaction is catalyzed by a microsomal NADPH-dependent reductase. Because 3-ketosphinganine is not detected in intact cells, it is suggested that the activity of the reductase is high *in vivo*. The product, sphinganine is then linked to a C_{26} fatty acid by a amide bond to give dihydroceramide. Ceramide synthesis apparently happens fast *in vivo* since free long-chain bases are not detected as intermediates in sphingolipid biosynthesis. This may be necessary because the free long-chain bases are potent inhibitors of protein kinase C and are cytotoxic. Metabolism of dihydroceramide in yeast is different than that in animals. In animals,

Figure 12. Sphingolipid biosynthesis *de novo* in yeast. The inositol-containing lipids are labeled according to their mobility on TLC performed as described under Materials & Methods.


ENDOPLASMIC RETICULUM
LUMEN
BAND B
Phosphatidylinositol
phytoceramide
BAND C
Diacylglycerol
sphinganine
3-Ketosphinganine
1
2
3
4
5
6
O_2
H_2O
Palmitoyl-CoA
Serine
NADPH
CO_2 · CoASH
dihydroceramide
inositolphosphorylphytoceramide
3'
7
CYTOSOL
C_{26} fatty acyl-CoA
Phytosphingosine
mannose-inositolphosphoryl
phytoceramide
O_2
9
8
inositolphosphorylmannose
-inositolphosphorylphytoceramide
OAG
PI
GDP
GOLGI LUMEN
BAND H
BAND G
BAND D
GDP-Mannose

dihydroceramide is oxidized to form ceramide and the choline-phosphate group is added from a phosphatidylcholine donor to form sphingomyelin. In *S. cerevisiae*, dihydroceramide is hydroxylated at C-4 of the long-chain base to form phytoceramide and inositol-P is added to the phytoceramide from a phosphatidylinositol donor. Inositolphosphorylphytoceramide is mannosylated in the Golgi by an enzyme using GDP-mannose as donor.

Little is known about the regulation of *de novo* biosynthesis of sphingolipid. Serine palmitoyl-transferase appears to catalyze the rate-limiting step since the intermediates formed before IPC formation are found at low levels.

C. The *in vivo* biosynthesis of sphingolipid is compartmentalized: As showed in Figure 12, the formation of ceramide backbone begins in the endoplasmic reticulum (ER) with the condensation of serine and palmitoylCoA. Serine palmitoyltransferase is located on the cytosolic side of the ER. Dihydroceramide synthesis may occur on the cytosolic surface of the endoplasmic reticulum (exposed to the phytoceramide precursors -- serine, palmitoyl-CoA, and C_{26}-fatty acyl CoA) and thus it is suggested that a transport protein (a flipase) is required to bring dihydroceramide into the endoplasmic reticulum lumen. Addition of the head group of sphingolipid (inositolphosphate and mannose) occurs in both the ER and Golgi apparatus. The addition of inositolphosphate group linked to the hydroxyl group derived from the serine side chain is likely to occur in the ER lumen. Several forms of inositolphosphoryl-phytoceramides that are separated by TLC apparently arise from

different levels of hydroxylation. One form, IPC-C, appears to be synthesized in the ER and is then converted to another form, IPC-D, in the Golgi (figure 12) since secretory (*sec*) mutants blocked at the ER --> Golgi step accumulate IPC-C, but do not make IPC-D, at the restrictive temperature [Puoti *et al.*, 1991].

In summary, in ER: serine + palmitoylCoA ---> inositolphosphorylphytoceramide, in Golgi: inositolphosphorylphytoceramide ---> mannose(inositolphosphoryl)$_2$phytoceramide.

D. Functions of sphingolipids [Lester and Dickson *et al.*, 1993]: In animals, sphingolipids are thought to play roles as modulators of membrane signal transducers, resulting in the regulation of cell growth and differentiation and as mediators of cell-to-cell or cell-to-substrates recognition [Hakomori, 1981]. In *S. cerevisiae*, sphingolipid is a vital lipid and is required for H^+-ATPase activity in plasma membrane which pumps protons out of the cell to create an electrochemical gradient that is utilized for transporting a variety of nutrients into the cell [Patton and Lester, 1992]. Sphingolipids also act as anchors for membrane proteins which form covalent attachment to inositolphosphorylceramide [Conzelmann *et al.*, 1992].

E. Dissection of the sphingolipid biosynthesis pathway in *S. cerevisiae*: Mutants that display absolute auxotrophy for exogenously added long-chain bases were selected in Lester's laboratory [Wells and Lester, 1983; Pinto *et al.*, 1992b]. They fall into two complementation groups which are named as *lcb1* and *lcb2*. Mutant strains

grew equally well with 3-ketodihydrosphingosine, erythrodihydrosphingosine or threodihydrosphingosine, or phytosphingosine which represent the first, second, and last components, respectively, of the long-chain-base biosynthetic pathway (Figure 12). When starved for long-chain bases, the mutant cells become denser than wild-type as evaluated by sedimentation on a gradient of Na ditriazoate [Pinto *et al.*, 1992b].

The *lcb1* mutant cells lack serine palmitoyltransferase (SPT) activity [Buede *et al.*, 1991], the first enzyme in the pathway for long-chain base synthesis. The wild type *LCB1* gene was cloned from a genomic library by complementation of the *lcb1* allele. The predicted amino acid sequence of the *LCB1p* shows high similarity to 5-aminolevulinic acid synthase (ALA synthase) and 2-amino-3-ketobutyrate CoA ligase. These two enzymes and SPT catalyze very similar chemical reactions involving decarboxylation of an α-amino acid with nucleophilic addition of the α-carbon to an acylCoA derivative. All of these enzymes use the cofactor pyridoxal phosphate (PLP). The cloned *LCB1* allele was able to restore SPT activity to a *lcb1*-defective strain. It was suggested that *LCB1* encoded SPT or a subunit of the enzyme.

Here a collection of mutants with altered sphingolipid metabolism is described. These mutants were isolated as the second-site suppressors of the Ca^{2+}-sensitive growth phenotype of *csg2* [Beeler *et al.*, 1994], and therefore are called Suppressor of Ca^{2+} Sensitivity (*scs*) mutants.

2. The *scs1* mutant acquires a Ca^{2+}-requiring phenotype:

As described in chapter three, all *scs1* mutant isolates display a Ca^{2+}-requirement for growth, suggesting that the mutated gene that causes suppression of *csg2* might play an important role in Ca^{2+} homeostasis. The EGTA-sensitive phenotype of the *scs1* mutant cells was used as a selection for cloning the wild type *SCS1* gene. The *scs1-1CSG2* mutant cells were transformed with a genomic library and cells that acquired the ability to grow on EGTA-YPD plates were isolated.

MATERIALS & METHODS:

Strains: The yeast strains used in this study are as described in Chapter Two and RW2907: *Mata his3-200 leu2= trp1-901 ura3-52 ade2-10*, RW2908: *Matα his3-200 leu2= trp1-901 ura3-52 ade5*, or were derived from them by standard crosses.

Plasmids: Plasmids are as described in Chapter Two. Plasmid pUC18 was used to construct the *scs1* knockout.

Media: Refer to Chapter Two.

Chemicals and Enzymes: Restriction endonucleases were obtained from either New England Biolabs Inc. or Gibco BRL Life Technologies. T4 DNA ligase was purchased from New England Biolabs Inc. HCl was supplied by Fisher Scientific Company. Chloroform was supplied by Mallinckrodt INC. Methanol was obtained

from J. T. Baker INC. L-[G-^{3}H]serine, $^{45}Ca^{2+}$, and α-[^{32}P]dATP were purchased from DuPont-New England Nuclear. The myo-[^{3}H]inositol was obtained from Amersham Life Science. All other chemicals were purchased from Sigma.

Yeast genetics: Sporulation materials (plates and liquid medium) were prepared according to Sherman *et al* [1974]. For tetrad dissection analysis, the sporulation was done on plates and the Singer micromanipulator was used to dissect the asci. For random analysis, the sporulation was done in liquid and the sporulated cultures were treated with ether to select against unsporulated cells [Campbell and Duffus (a), 1988]. The unsporulated diploids were killed by a 4-minute treatment with an equal volume of dimethyl ether. The spores were released from the asci by vortexing with one-third volume of acid washed glass beads (0.5 mm diameter) for 90 seconds (3 X 30s) at 4°C.

Determination of the growth (or death) rate of cells under various conditions:

(1) By following the change in OD_{600} with time in liquid media: Cells were inoculated in their permissive media-- wild type and *csg2Δ* strains in YPD medium at pH 4.7 while *scs1-1/csg2* and *scs1-2/csg2* strains in YPD + 100 mM Ca^{2+} medium at pH 4.7. Cells were harvested in the logarithmic phase by centrifugation and resuspended at an OD_{600}= 0.05 in YPD, YPD + 1 mM Ca^{2+}, YPD + 3 mM Ca^{2+}, YPD + 10 mM Ca^{2+}, YPD + 20 mM Ca^{2+}, YPD + 50 mM Ca^{2+} and YPD + 100 mM Ca^{2+} . At time = 0, 4, 8, and 12 hr, aliquots were removed and the OD was

read in the presence of 0.25 M of EDTA at pH 8.0. To determine the growth rate the ln(OD) was plotted against the time. The growth rate was determined by the slope of the lines.

(2) By following the number of cells capable of forming colonies with time: Cells were grown overnight in rich media to O.D.= 0.1 to 0.4. The cells were washed with the test media at 4°C once and resuspended in the test media at 26°C. At time 0, 15, 30, 60, 120, 240 min, 0.5 ml of cells was removed and diluted (2500-fold). One hundred microliters were plated onto YPD or YPD + 100 mM Ca^{2+} plates. After two days' incubation at 26°C, the colonies were counted.

Acid titration: To determine the membrane permeability to protons, *scs1-1* cells were titrated with acid (HCl) as described by Patton *et al* [Patton *et al.*, 1992]. The cultured cells were washed with 1 M sorbitol. The titration was carried out at 30°C with compressed air bubbling through the suspension continuously. One micromolar of HCl was added to the suspension every 30 seconds. The changes of pH were measured by a pH meter (CORNING, pH meter 140). The permeablized cells were obtained by incubating the cells in 10 ml of 500 mM NaCl-9% n-butanol at 40°C for 5 min.

Nucleic acid manipulation:

1. The preparation of high molecular weight DNA used for Southern blot analysis and for generation of genomic DNA for transformation into *E. coli.* to

recover plasmid was based upon the method of Struhl *et al* [1979].

2. Plasmid DNA was prepared from *Escherichia coli* by a modification of the method of Holmes and Quigley [1981] as described in Chapter Two.

3. The yeast cells were cultivated, treated and transformed as described in Chapter Two.

4. Sequencing reactions were performed by both the dye primer reaction using M13mp18 and mp19 templates and the dye terminator reaction using synthetic oligonucleotides as primers. Sequencing reactions were analyzed as described in Chapter Two. The entire sequence was determined for both strands.

SCS1 Gene Cloning: The *SCS1* gene was cloned by complementation of the Ca^{2+}-requiring phenotype of the *scs1-1* mutant. A *Matα scs1-1 CSG2*$^+$ *lys2 ura3-52 trp1Δ leu2Δ* strain was used to select the wild type *SCS1* gene from the YCp50-based genomic library of Rose *et al.* [1987]. Transformants were selected first for uracil prototropy and subsequently by replica plating to YPD + 10 mM EGTA. The *scs1-1 CSG2* mutant does not grow on YPD plates supplemented with 10 mM EGTA. One EGTA-resistant colony was found out of 7500 transformants. When cells were grown on high Ca^{2+} plates to allow plasmid segregation, the resulting ura$^-$ segregants (selected on 5-fluoroorotic acid plates [Boeke *et al.*, 1984]) simultaneously reverted to the Ca^{2+}-requiring phenotype showing that the complementing gene was plasmid-linked.

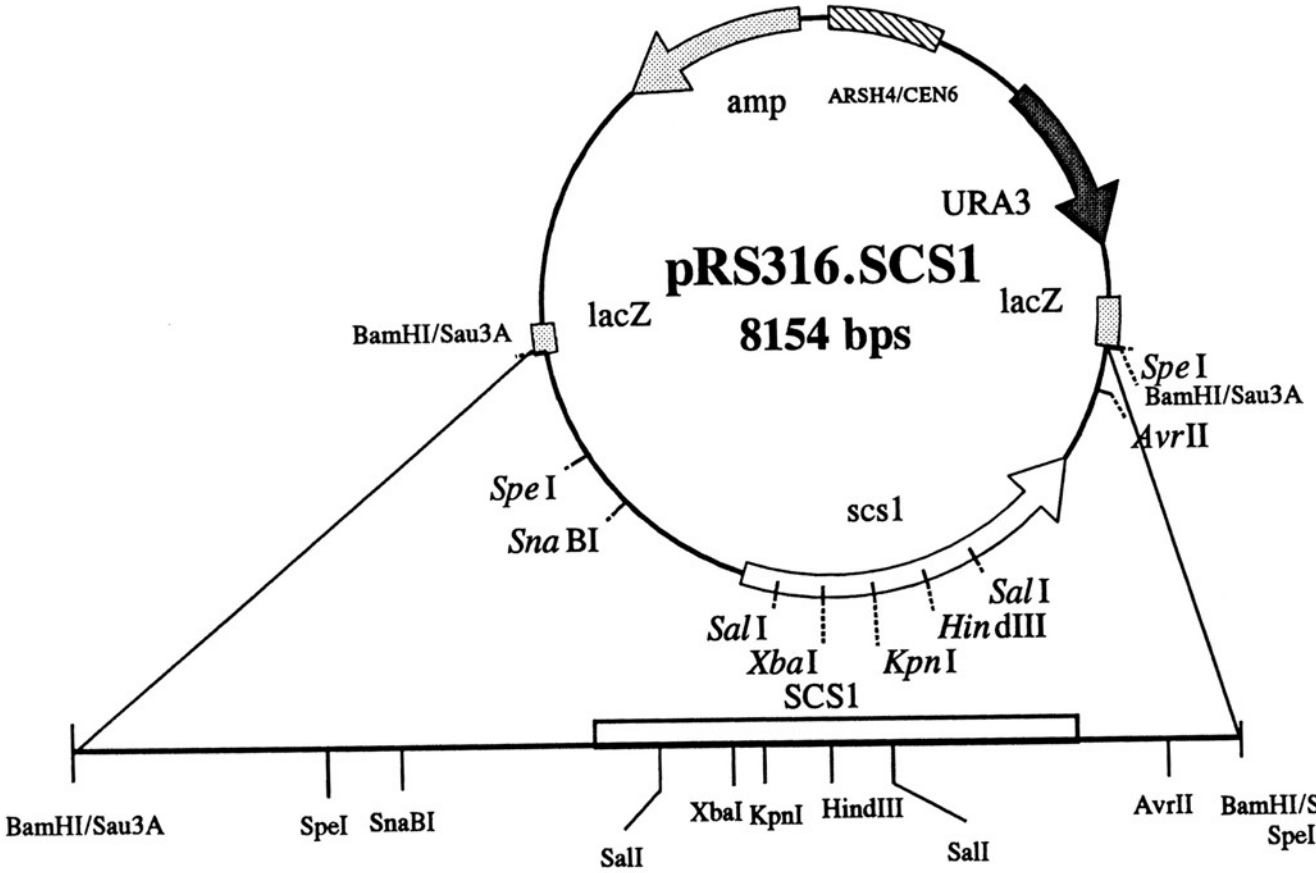
The linkage of the cloned DNA to the *SCS1* locus was demonstrated by

marking the locus of the cloned gene with *URA3*$^+$ and determining the segregation of the *URA3*$^+$ and *scs1-1* phenotypes following meiosis. An integrating plasmid was constructed by subcloning the 3164 bp *SpeI* fragment of the complementing plasmid into pRS306. The resultant plasmid was cut with *AvrII* and used to integratively transform a wild type *SCS1* strain thereby marking the chromosomal locus homologous to the insert with the *URA3*$^+$ gene. A *URA3*$^+$ transformant was mated to an *scs1-1* haploid, and the resulting diploid was sporulated. In all 12 tetrads analyzed, parental ditype segregation of the Ca^{2+}-requiring phenotype and the *URA3*$^+$ phenotype was observed, indicating that the cloned fragment included the wild type *SCS1* gene.

Localization of the SCS1 gene: *Sau3A* partial digest fragments of the complementing plasmid were subcloned into a CEN-plasmid (pRS316) and tested for ability to complement *scs1-1 CSG2* cells. All complementing plasmids contained the 2670 bp *SnaBI* to *AvrII* fragment (Figure 13). Since the *SpeI* fragment complemented the *scs1-1* phenotype, while the *SpeI-HindIII* and *XbaI-SpeI* fragments did not, it was concluded that the *HindIII* and *XbaI* sites lie inside the *SCS1* gene.

Construction of the scs1 null mutant: A null allele of *SCS1* in which 304 amino acids of the *SCS1* coding sequence was replaced with the *LEU2*$^+$ gene was generated by inserting the *AvrII/SnaBI* fragment containing the *SCS1* gene into the *XbaI/HincII* sites of pUC18. The *SalI* fragment in the *SCS1* coding region (Figure

Figure 13. Localization of the *SCS1* gene on the complementing plasmid. Plasmids carrying the indicated fragments were tested for their ability to complement the *scs1-1* Ca^{2+}-requiring phenotype.



	Complements
———————————	YES
———————	NO
———————————	NO

13) was replaced by the 2200 bp *LEU2*$^+$ fragment to give the *SCS1*-disrupting plasmid. The 4000 bp *BamHI/HindIII* fragment from this plasmid was transformed into wild type diploid cells. *LEU2*$^+$ transformants were selected on SD plates and disruption of the *SCS1* gene was confirmed by Southern analysis [Southern, 1975].

Southern blot analysis: All of the Southern blots were done as described [Southern, 1975]. Probes were labeled using the random primers DNA labeling kit from Bethesda Research Laboratory (BRL).

Serine palmitoyltransferase assay: Cells were grown to an OD_{600} of 4.0-6.0 in YPD + 25μM phytosphingosine (pH 4.7) since our previous experiment data showed that there was no difference in serine palmitoyltransferase activity between wild-type cells cultured with or without phytosphingosine in the media. Membranes were prepared using the procedure described by Lester and co-workers [Pinto *et al.*, 1992a] with one modification. The final membrane fraction was layered on 40% sucrose and centrifuged at 100,000 g for 30 min. Membranes at the 0-40% sucrose interface were removed, concentrated by centrifugation (100,000 g for 30 min) and stored at -20°C in 25% glycerol. Protein concentration was assayed by use of the dye-binding method of Bradford [1976]. The enzyme assay was performed using the procedure described by Pinto *et al.* [1992a]. The reaction was performed at 30°C for 20 min with shaking in 0.2 ml of 0.1 M HEPES (pH 8.3), 5 mM dithiothreitol, 2.5 mM EDTA, 50 μM pyridoxal phosphate, 40 μM palmitoyl CoA, 5 mM L-serine, L-[G-^{3}H]serine (5 μCi),

and 0.2 mg of membrane protein. The reaction was terminated with 0.5 ml of 0.5 N NH_4OH containing 5 μmol of L-serine. The labeled inositol-containing lipids were extracted by chloroform in the presence of 50 μg of 3-KDS. The radioactivity was measured on a Packard 1500 TRI-CARD liquid scintillation analyzer. Radioactivity in the control without enzyme was subtracted from all of the assays to calculate the specific activity.

Analysis of Inositol-containing Lipids: Inositol-labeling, extraction and silica gel thin layer chromatography analysis of the sphingolipids was done according to Robbins and co-workers [Abeijion *et al.*, 1993]. The cells were grown in inositol-free SD medium. Cells were suspended at 10 OD_{600}/ ml in SD containing 0.5 μM [^{3}H]-inositol (50 μCi/ ml), with and without 100 mM $CaCl_2$ and incubated for 10 min. The cells were then diluted 4-fold in SD medium containing 300 μM unlabeled inositol with or without 100 mM Ca^{2+}. After a 90 min incubation, the lipids were extracted from the cells according to Abeijon *et al.* [1993]. Cells were pelleted, resuspended in 1 ml SD + 3 mM NaN_3 and repelleted. The pellet was suspended in 760 μl of $H_2O/CHCl_3/CH_3OH$ (3:10:10). Glass beads (0.5 mm diameter, acid washed) were added to the meniscus and cells were disrupted by vortexing for several minutes. The supernatant was removed and the beads were reextracted with 760 μl of $H_2O/CHCl_3/CH_3OH$ (3:10:10). The pooled supernatants were dried and the residue was resuspended in n-butanol and desalted according to Krakow *et al.* [1986]. Alkaline treatment was performed according to Becker and Lester [1980]. The

sample was dissolved in 200 μl of $CH_3CH_2OH/H_2O/CH_3CH_2OCH_2CH_3$/pyridine (15:15:5:1) and 200 μl of 0.2 M KOH in CH_3OH for 1 hour at 27°C. Forty microliter of 1 N acetic acid was added, samples were dried, and desalted. Samples were spotted on silica gel thin layer chromatography plates and developed in $CHCl_3/CH_3OH$/0.25% KCl in water (55:45:10).

RESULTS:

1. The same mutation confers both the suppression and the EGTA-sensitive phenotypes of the *SCS1* mutant:

Tetrad analysis of a sporulated *scs1-1 csg2 / SCS1 csg2* diploid yielded two Ca^{2+}-resistant haploids per tetrad, demonstrating that the suppressor phenotype results from a single gene mutation. Furthermore, the Ca^{2+}-requiring and *csg2*-suppressing phenotypes were completely linked, demonstrating that the same mutation confers both phenotypes.

Random spore analysis of diploids *scs1-1 csg2::LEU2*$^+$*/ SCS1 CSG2* and diploids *scs1-2 csg2::LEU2*$^+$*/ SCS1 CSG2* showed the ratio among the spores of Ca^{2+}-requiring *LEU2*$^+$ to Ca^{2+}-requiring *leu2*$^-$ to Ca^{2+}-sensitive *LEU2*$^+$ to Ca^{2+}-resistant *leu2*$^-$ was 1:1:1:1. This also confirmed that the *scs1* mutation is a single gene mutation and the mutation confers both Ca^{2+}-requiring and Ca^{2+}-suppressing phenotypes. Furthermore, it demonstrated that the *scs1-1 CSG2*$^+$ haploid is Ca^{2+}-requiring. The strain *Matα scs1-1 CSG2*$^+$ *ade2-101 leu2Δ ura3-52 trp1Δ* was selected from spores obtained.

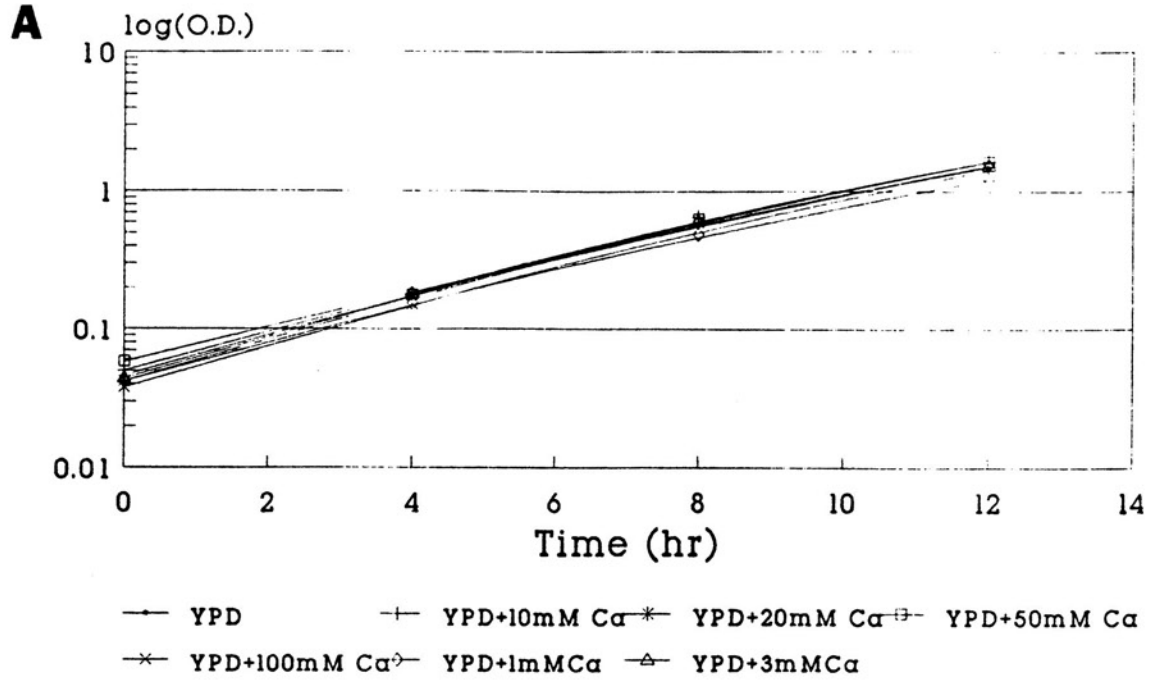
2. The *scs1* cells require Ca^{2+} not only for their growth but also for maintenance of viability:

As described in Chapter 3, *scs1-1* cells acquire a requirement for increase Ca^{2+} concentration for growth on YPD plates. A similar Ca^{2+} requirement was found for cell growth in liquid YPD medium (Figure 14). The *scs1-1* cells require about 10 mM Ca^{2+} for growth. Growth was measured by monitoring the change in OD_{600nm}. The rate of wild type cell growth was not effected by 0.3-100 mM Ca^{2+} (Figure 14A). Whereas, the growth of *csg2Δ* cells was inhibited by 10 mM Ca^{2+} [Beeler *et al.*, 1994]. In contrast, the *scs1-1* cells (Figure 14B) required 10 mM or higher Ca^{2+} for growth.

Viability of *scs1-1* mutant cells can be measured by incubating the cell in YPD with varying Ca^{2+} concentration and plating aliquots on YPD + 100 mM Ca^{2+} plates at different times to determine the number of viable cells. The growth rate (or death rate) was plotted versus different growth media (Figure 15A). Wild-type cells did not vary in media containing 0.3-100 mM Ca^{2+}. The rate of cell death upon transfer of *scs1-1* mutant cells from YPD + 100 mM Ca^{2+} to YPD is relatively rapid with a half-time of about 25 min. Addition of 0.6 M sorbitol slows the rate of death by about 25% (Figure 15B), but does not prevent it indicating that death is not simply due to osmotic-induced cell lysis. The rate of cell death is dependent on the Ca^{2+} concentration in the range of 0 to 10 mM. The *scs1-1 CSG2* cells are more tolerant of low Ca^{2+} than the *scs1-1 csg2* cells, since in YPD medium, they had a longer life-time (i.e., a longer half-life for death). Furthermore, 3 mM Ca^{2+} prevented their death although it did not prevent the death of *scs1-1 csg2* cells. Thus the Ca^{2+}-

Figure 14. Effect of Ca^{2+} on the growth rate of wild-type and *scs1-1* mutant cells. *Panel A*, wild-type cells were grown in YPD buffered to pH 4.7 with citrate at 26°C to an OD_{600} of 0.5. *Panel B*, the *scs1-1* mutant cells were grown in the same conditions as wild-type cells except that 100 mM $CaCl_2$ was added to the YPD medium. The cells were then split to YPD, pH 4.7, with or without Ca^{2+}. At the indicated time aliquots were removed, diluted 1:1 in 0.5 M K-EDTA, pH 7.5, and the OD_{600} was determined. Dilutions were made as necessary to maintain the OD_{600} between 0.1 and 0.5 through the experiment. The slope of each line represents the growth rate = $(\ln(OD)_1 - \ln(OD)_2)/ (T_1 - T_2)$.

2039 (wt)



9R (scs1-1)

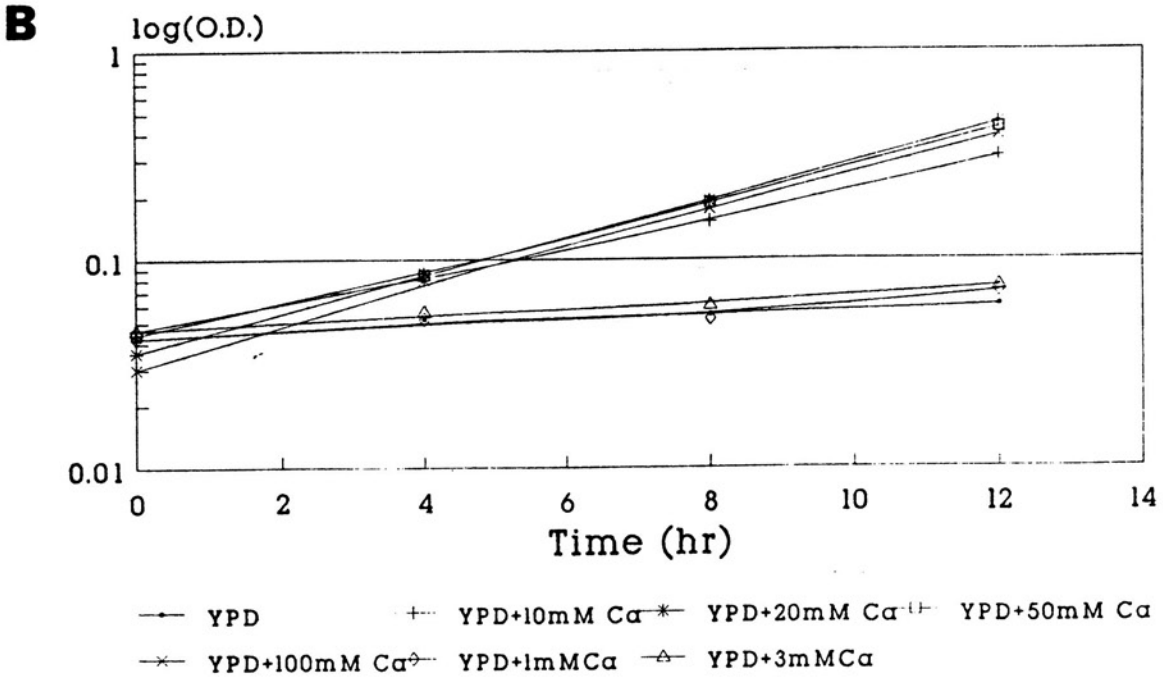
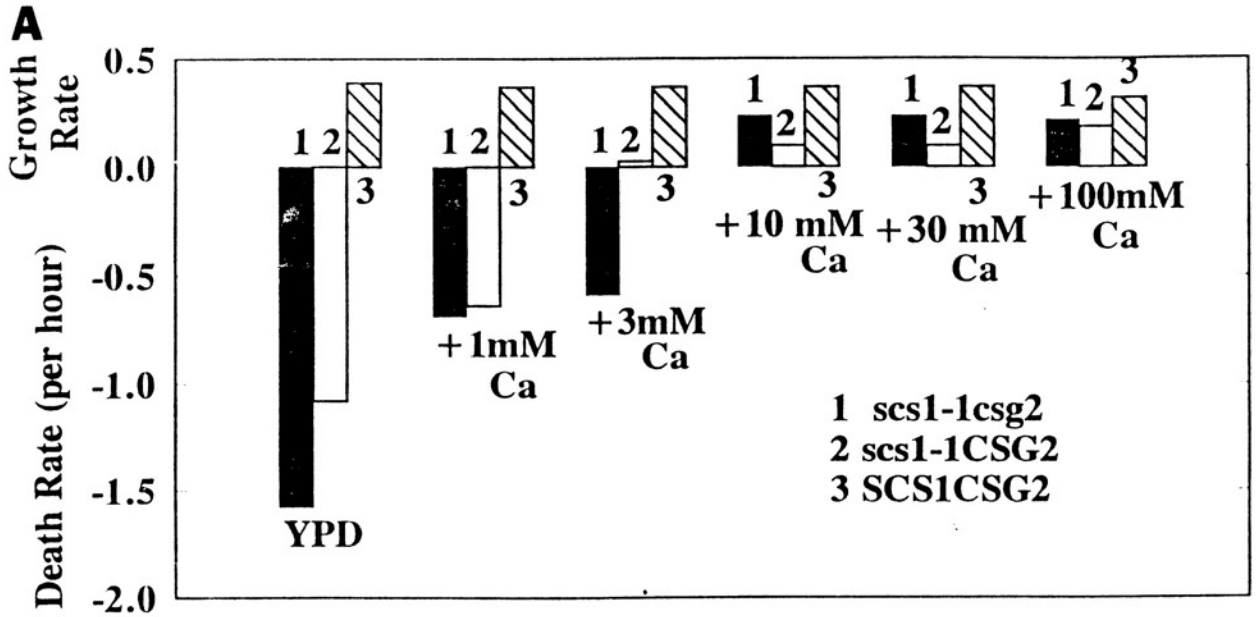
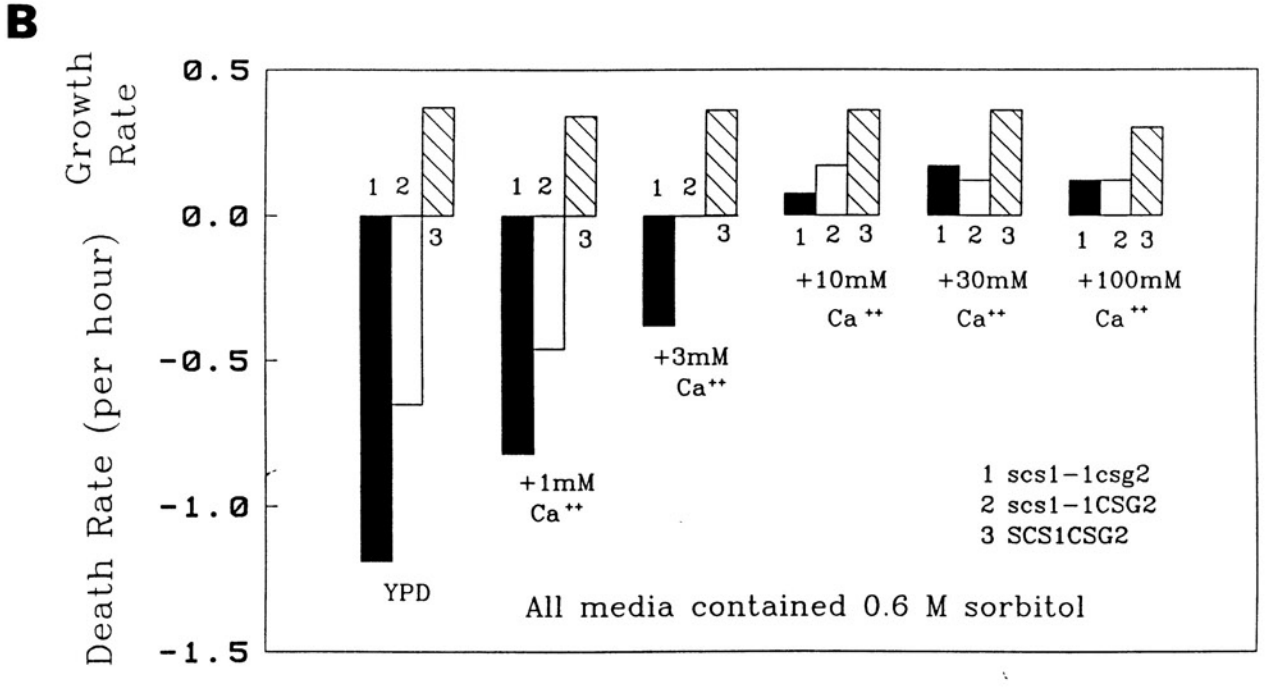


Figure 15. Effect of Ca^{2+} on the viability and rate of cell growth. *Panel A*, the *scs1-1 csg2*, *scs1-1 CSG2*, and *SCS1 CSG2* strains were grown in YPD + 100 mM $CaCl_2$ to an OD_{600} of 0.2-0.8. Cells were washed by centrifugation with YPD + the indicated $CaCl_2$ concentration and resuspended in the same. At time 0, 15, 30, 60, 120, and 240 minutes aliquots were removed, diluted, and plated on YPD + 100 mM $CaCl_2$ to determine cell viability. Growth or death rates are calculated from the slope of the line formed when ln(cell number) is plotted against time. Rate = $(\ln(\text{cell number}_1) - \ln(\text{cell number}_2))/ (T_1 - T_2)$. *Panel B*, the same as panel A except that 0.6 M sorbitol was added to all media.


A
Growth Rate
Death Rate (per hour)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
1 2 3
YPD
+1mM Ca
+3mM Ca
+10 mM Ca
+30 mM Ca
+100mM Ca
1 scs1-1csg2
2 scs1-1CSG2
3 SCS1CSG2


B
Growth Rate
Death Rate (per hour)
0.5
0.0
-0.5
-1.0
-1.5
1 2 3
YPD
+1mM Ca++
+3mM Ca++
+10mM Ca++
+30mM Ca++
+100mM Ca++
1 scs1-1csg2
2 scs1-1CSG2
3 SCS1CSG2
All media contained 0.6 M sorbitol

requiring phenotype is tighter (or lower reversible rate) in combination with the *csg2Δ* mutant than with wild type *CSG2*$^{+}$.

Cells in logarithmic growth stop growing when they are transferred to media without glucose. When the *scs1-1* cells were incubated in YP + 100 mM Ca^{2+} medium for two hours, the Ca^{2+} requirement of non-growing cells were determined by incubating the cells in YP media (without glucose) and with or without 100 mM Ca^{2+} for two hours and plating aliquots on YPD + 100 mM Ca^{2+} plates to determine the number of viable cells. It was found that even the non-growing *scs1-1* cells required Ca^{2+} for their viability, which suggested that Ca^{2+} is required to maintain membrane integrity.

3. The observation that cellular Ca^{2+} levels are high in *scs1-1* cells grown in 100 mM Ca^{2+} suggested that the Ca^{2+} requirement is not due to a block in the cellular Ca^{2+} influx:

Because *csg2* mutants had increased cellular Ca^{2+} accumulation, one might expect that *scs1-1csg2* mutants would have a decreased rate of Ca^{2+} accumulation and therefore become Ca^{2+} requiring.

In order to determine whether the *scs1* Ca^{2+} requirement arises from a decreased rate of cellular Ca^{2+} uptake, the rate and the level of $^{45}Ca^{2+}$ accumulation by *scs1-1* cells was measured by filtration of cells followed by radioactive counting. Ca^{2+} uptake rate and Ca^{2+}-loading level were much higher in *scs1-1csg2* compared with wild type or *csg2Δ* in YPD medium (nonpermissive for growth of *scs1*) (Figure

16A). When the YPD Ca^{2+} concentration was increased to 10 mM (which blocks *scs1-1* cell death (Figure 15A)), cellular Ca^{2+} accumulation was even higher (Figure 16B). It is interesting that *scs1-1* cells grown in YPD + 100 mM Ca^{2+} accumulated less Ca^{2+} (1101 nmol Ca^{2+}/mg protein) than cells incubated for 1 hour in YPD (0.3 mM Ca^{2+}, 3200 nmol Ca^{2+}/mg protein). The data suggested that the *scs1-1* mutant permits increased Ca^{2+} flux into the cell.

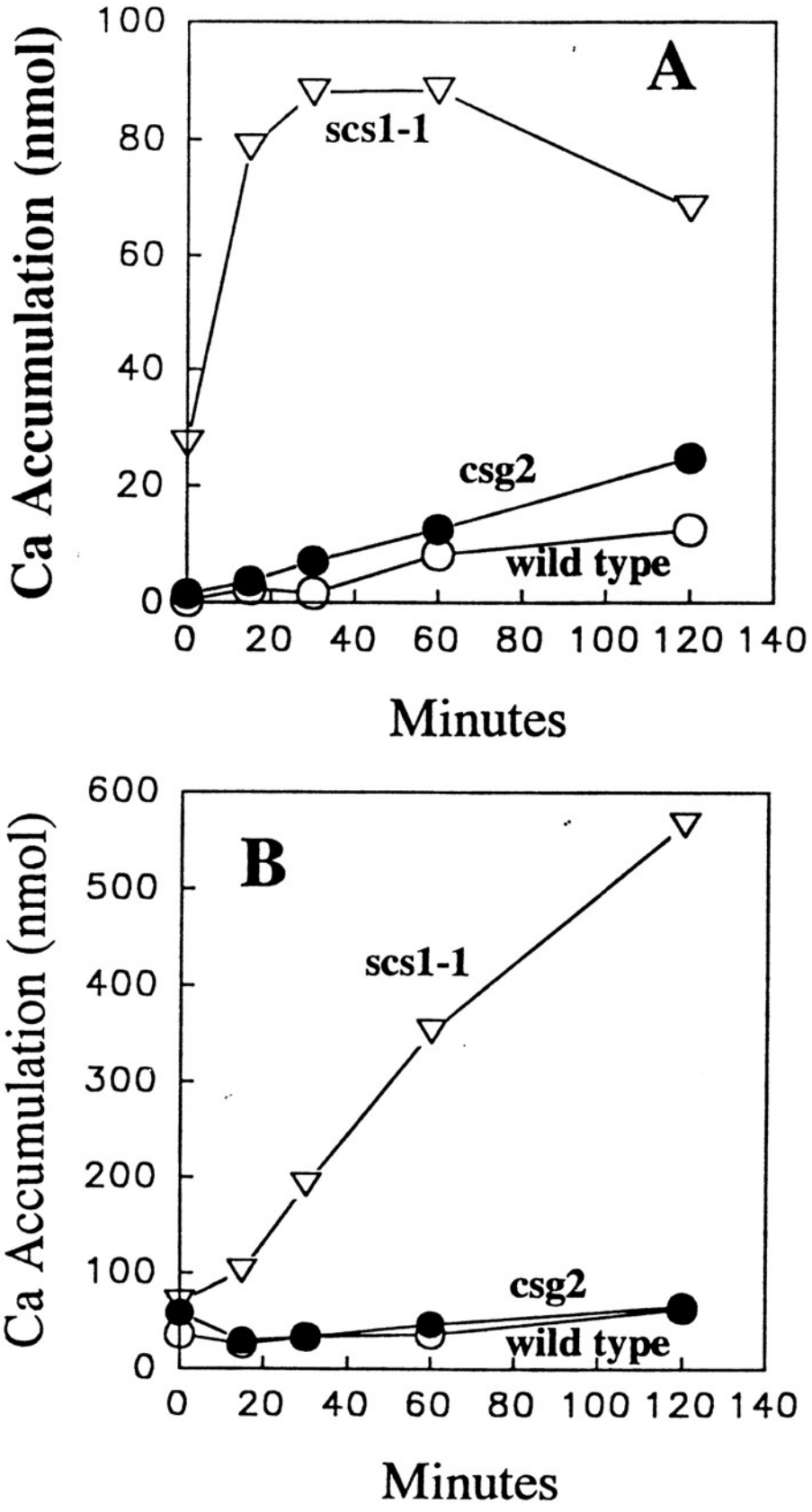
4. Cloning of *SCS1*:

The wild type *SCS1* gene was isolated from a genomic library by complementation of the Ca^{2+}-requiring phenotype of the *scs1-1* mutant. The *scs1-1 CSG2* strain was transformed with a YCp50-based *S. cerevisiae* genomic library. The *scs1-1 CSG2* strain was used instead of *scs1-1 csg2* because the former has a higher transformation efficiency. About 7500 transformants were selected on SD plates without uracil. After transferring the transformed cells to YPD + 10 mM EGTA plates, one transformant had acquired EGTA-insensitivity (Ca^{2+} independence). EGTA was added to the YPD to make the medium less permissive thereby decreasing the reversion frequency; the growth of *scs1-1 CSG2* is more permissive on YPD than that of *scs1-1 csg2*. The complementing plasmid carries the gene that allows growth of *scs1-1* on YPD + 10 mM EGTA since cells that lose the plasmid simultaneously become Ca^{2+}-requiring.

The *SCS1* gene was localized by subcloning fragments of the insert and testing for their ability to confer growth on YPD + 10 mM EGTA. *SCS1* was mapped to a

Figure 16. Rate of Ca^{2+} accumulation by *scs1-1 csg2, SCS1 csg2* and *SCS1 CSG2* stains.

The *scs1-1 csg2* and *SCS1 CSG2* strains were grown in YPD + 10 mM Ca^{2+} to an OD_{600} of about 0.5. The *SCS1 csg2* strain was grown in YPD and transferred to YPD + 10 mM Ca^{2+} two hours before the start of the Ca^{2+} uptake measurements. The cells were washed with YPD at 4 °C by centrifugation to remove excess Ca^{2+}. In panel A, cells were resuspended to OD_{600} = 0.25 in YPD containing 1 μCi/ ml $^{45}Ca^{2+}$. In panel B, cells were resuspended to OD_{600} = 0.25 in YPD + 10 mM $CaCl_2$ containing 1 μCi/ ml $^{45}Ca^{2+}$. At the indicated times, 1 ml aliquots were filtered, the filters were washed three times with 5 ml of 20 mM $MgSO_4$ (4 °C) and dried. The cell-associated $^{45}Ca^{2+}$ was measured by scintillation counting. Aliquots were also plated for cell viability. During the time course of the experiment, in panel A condition, the *scs1-1 csg2* cells died with a half-time of 25 min, while *SCS1 CSG2* and *SCS1 csg2* stains grew with a doubling time of 2.1 and 2.0 hours respectively; whereas in panel B condition, the doubling time of the *scs1 csg2, SCS1 csg2*, and *SCS1 CSG2* cells were 4.9 h, 2.2 h, and 2.4 h respectively.


A
scs1-1
csg2
wild type
Ca Accumulation (nmol)
100
80
60
40
20
0
0 20 40 60 80 100 120 140
Minutes
B
scs1-1
csg2
wild type
Ca Accumulation (nmol)
600
500
400
300
200
100
0
0 20 40 60 80 100 120 140
Minutes

3164 bp *SpeI* fragment and the *HindIII* and *XbaI* sites within this fragment were found to reside in the *SCS1* gene. This region was sequenced and an open reading frame of 1683 bp which encodes a protein of 561 amino acids was found (figure 17).

Southern blot analysis of the contiguous clones of the yeast genome using the 3164 bp *SpeI* fragment as a probe localizes the *SCS1* gene on the right arm of chromosome IV between the *PMR2* and *CDC34* genes [Link and Olsen, 1991].

5. Analysis of the amino acid sequence of SCS1p:

A search of the protein sequence databases identified significant homology between *SCS1p* and aminolevulinate synthetase (HEM1) [Urban-Grimal *et al.*, 1986], 7-keto-8-aminopelargonic acid synthetase (BIOF) [Gloeckler *et al.*, 1990], α-amino-β-ketobutyrate synthetase (KBL) [Aronson *et al.*, 1988], and serine palmitoyltransferase (LCB1) [Buede *et al.*, 1991] (Figure 18). This family of pyridoxal phosphate-utilizing enzymes catalyzes the transfer of an acyl group from an acyl-CoA substrate to the α-carbon of an amino acid (glycine, alanine or serine). A consensus pyridoxal phosphate binding site containing the lysine that forms the Schiff base linkage has been identified [Mukherjee and Dekker, 1990]. The members of this acyl transferase family (except LCB1p) have the following consensus sequence around this lysine position:

(D/E)X(I/L)XX(T/S)(L/F)(G/T)**K**X(L/F)GXX(G/S)(G/A)

The homologous region in SCS1p is 362 GTFT**K**SFG 369 (Figure 18). In LCB1p a threonine is found in place of the lysine. Since the lysine is expected to be

Figure 17. Nucleotide and deduced amino acid sequence of *SCS1*. The sequence was determined as described under "Materials & Methods".

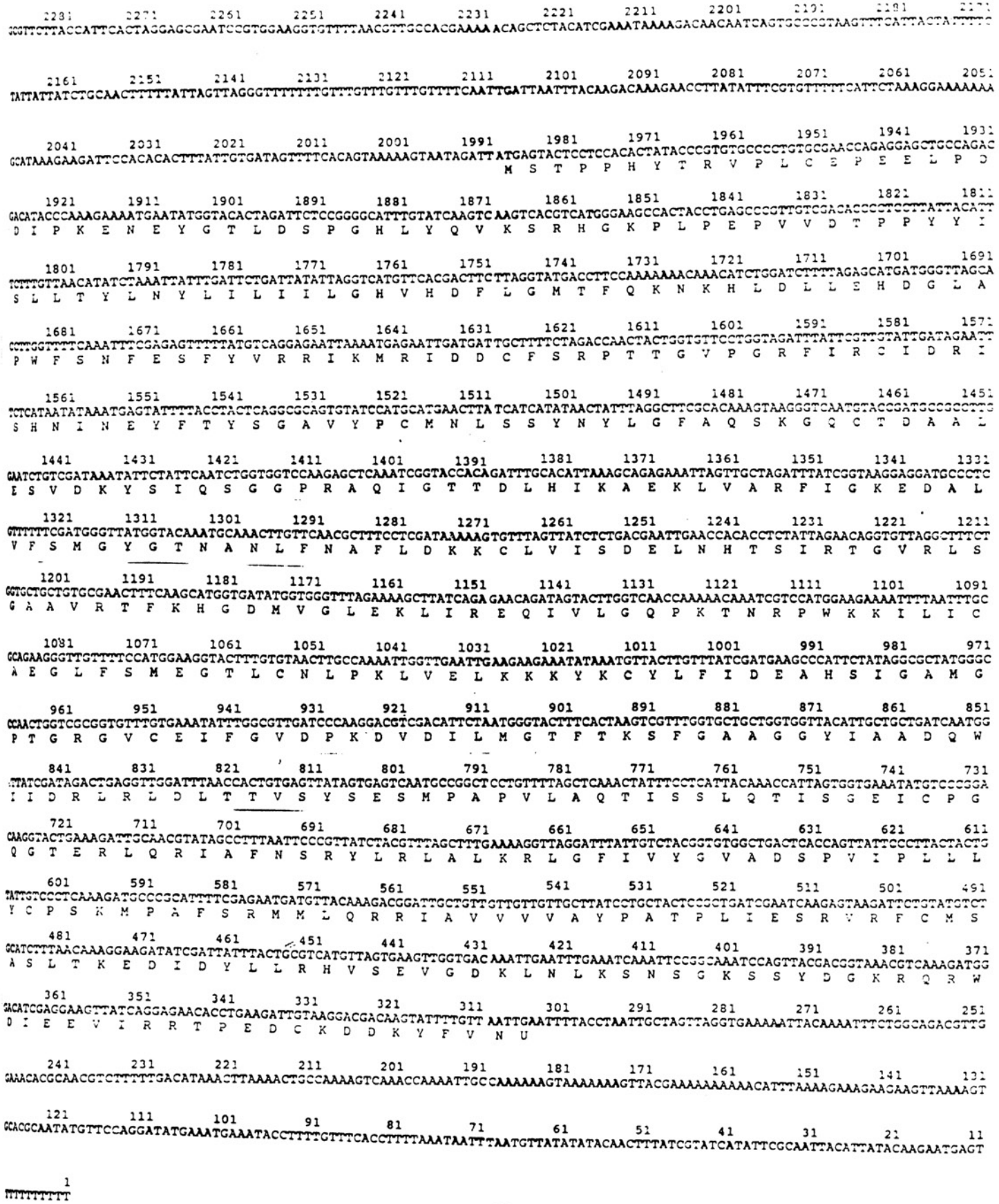
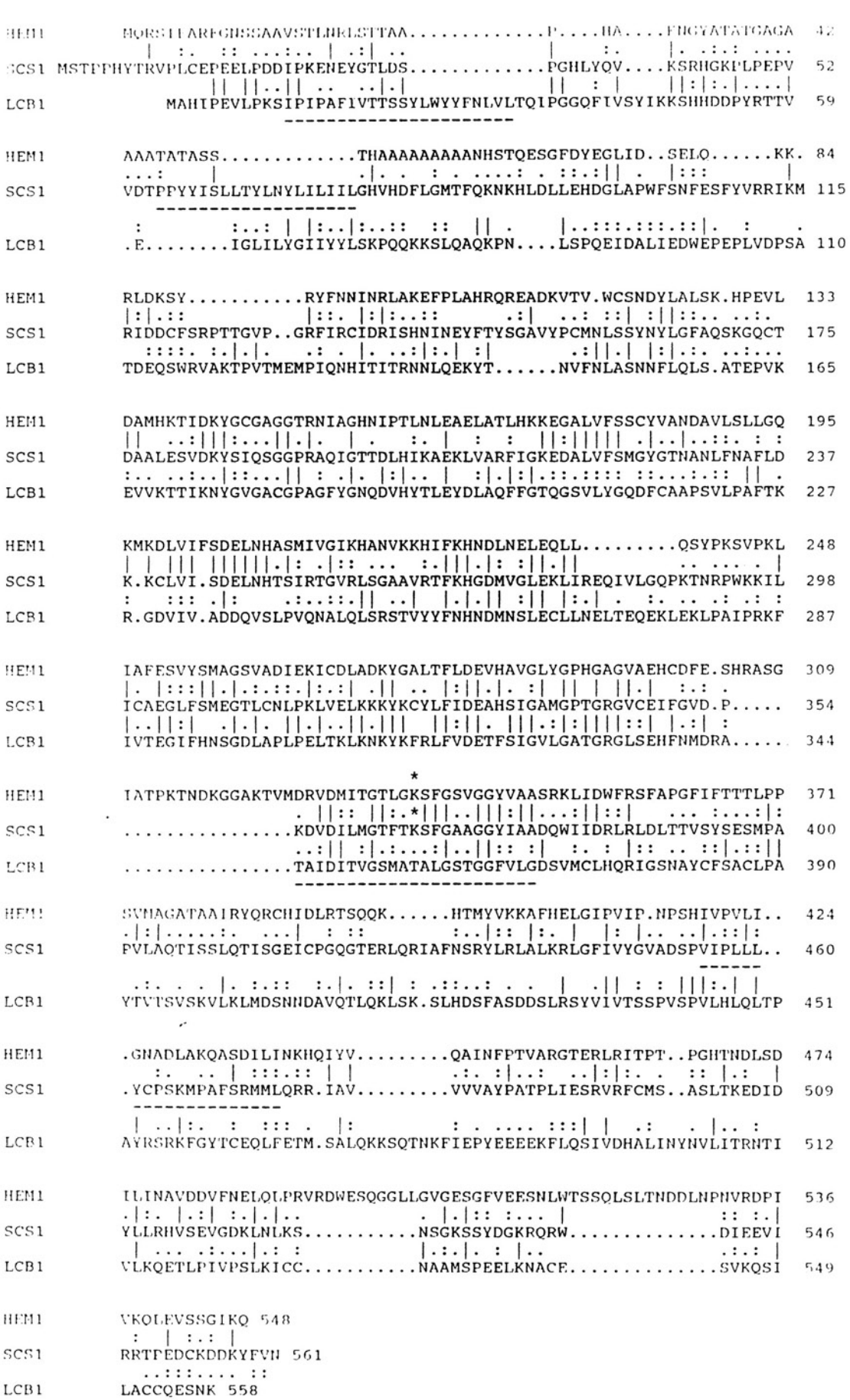


Figure 18. Comparison of the amino acid sequences of yeast HEM1p, LCB1p and SCS1p. Hydrophobic segments in SCS1p and LCB1p are underlined. The lysine in the consensus sequence for pyridoxal phosphate binding is indicated by an asterisk. Amino acid alignment was according to the BESTFIT program of the GCG package.


HEM1
SCS1
LCB1

required for catalysis, its absence from LCB1p suggests that LCB1p may not be the catalytic subunit of serine palmitoyltransferase.

The region of SCS1p that shows homology to the acyltransferase family extends from amino acid 175 to amino acid 483. This region is flanked by two hydrophobic regions (56PPYYISLLTYLNYLILIILG76 and 453VIPLLLYCPSKMPAFSRMML469). These hydrophobic segments do not clearly define transmembrane segments since their average hydrophobicity is only 1.6 and 1.0 respectively [Kyte and Doolittle, 1982]. Transmembrane segments typically show averages of 2 and seldom are below 1.2.

6. Identification of *SCS1* as a serine palmitoyltransferase gene:

The function of SCS1p could not be deduced from its homology with the other pyridoxal phosphate-containing acyl transferases since SCS1p showed comparable similarity (about 40%) to all members of the family. However it was unlikely that SCS1 encodes 7-keto-8-aminopelargonic acid synthetase (an enzyme required for biotin synthesis) because yeast do not synthesize biotin *de novo*. Nor was it likely to be α-amino-β-ketobutyrate synthetase (involved in threonine breakdown), because this enzyme is not expected to be essential, whereas the *SCS1* is essential, as presented later. Addition of aminolevulinate to the medium bypasses the need for aminolevulinate synthetase but did not alter the *scs1* phenotype, indicating that aminolevulinate synthetase is not defective in the mutant. However, phytosphingosine, which bypasses the requirement for serine palmitoyltransferase

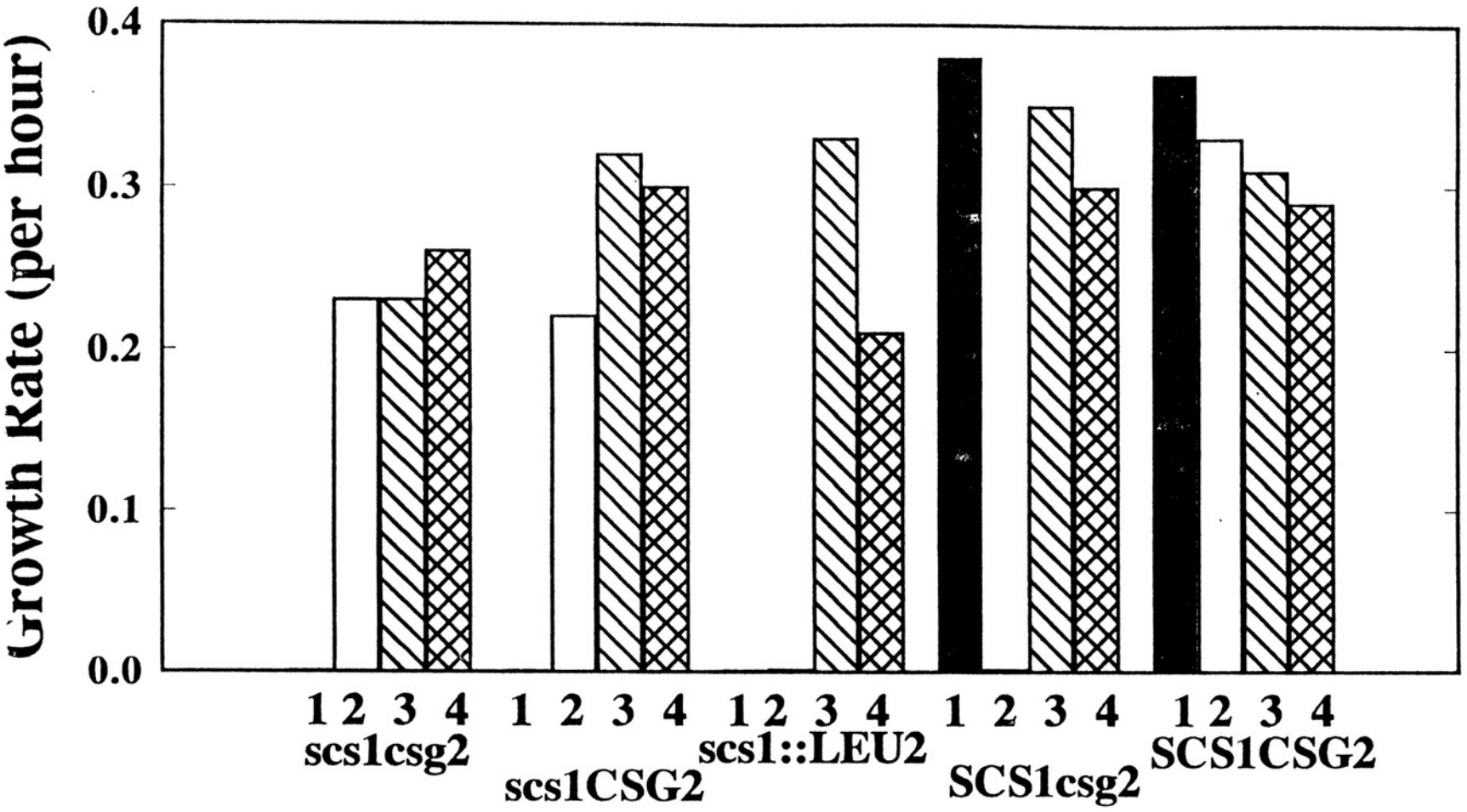
[Buede *et al.*, 1991], did reverse the Ca^{2+}-requiring phenotype of *scs1-1*, suggesting that *SCS1* may encode serine palmitoyltransferase or a subunit thereof (Figure 19). Wild type cells (*SCS1 CSG2*) grew in all four media. The *scs1 csg2* and *scs1 CSG2* strains grew in YPD + 100 mM Ca^{2+} medium but not in YPD. When 3 μM phytosphingosine was added, the *scs1 csg2* and *scs1 CSG2* grew in both YPD and YPD + 100 mM Ca^{2+} media.

When testing whether phytosphingosine also reverses the ability of *scs1* to suppress *csg2*, it was observed that 3 μM phytosphingosine alone reverses the Ca^{2+}-sensitivity of *csg2*; i.e., *csg2* mutant cells grow in YPD + 100 mM Ca^{2+} in the presence phytosphingosine but they did not grow without phytosphingosine (Figure 19). Dihydrosphinganine (5 μM) also restored the ability of the *scs1-1* mutant cells to grow in YPD medium and reversed the *csg2* Ca^{2+}-sensitivity. These observations suggested that the Ca^{2+}-sensitive *csg2* mutant phenotype may arise from alterations in sphingolipid biosynthesis or metabolism.

7. The *scs1-1* cell membrane is not permeable to protons:

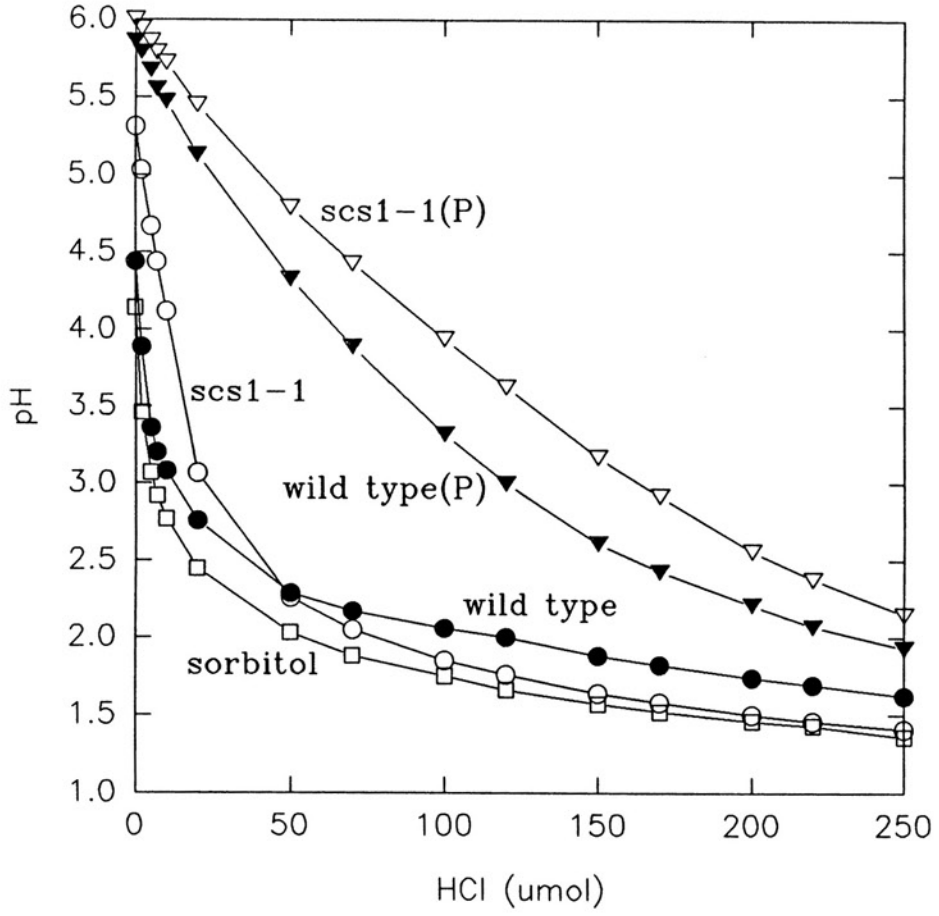
Lester and coworkers found that *lcb1* cells lacking sphingolipid appeared to become rapidly permeable to protons at low pH [Patton *et al.*, 1992]. Proton permeability was measured by the ability of cell to buffer the extracellular medium. It was of interest to test the response of *scs1-1* cells to protons. The rapid acidification of the medium by *scs1-1* cells exposed to low pH (Figure 20) suggested that the cells may not be permeable to protons, which means that the external pH

Figure 19. Effect of phytosphingosine on the growth rate of *scs1 csg2, scs1 CSG2, scs1::LEU2*$^+$ *CSG2, SCS1 csg2* and *SCS1 CSG2*. Cells were grown in either YPD (*SCS1 CSG2* and *SCS1 csg2*), YPD + 100 mM Ca^{2+} (*scs1-1 csg2, scs1-1 CSG2*) or YPD + 25 μM phytosphingosine (*scs1::LEU2*$^+$ *CSG2*). The cells were then diluted into YPD (1), YPD + 100 mM $CaCl_2$ (2), YPD + 3 μM phytosphingosine (3), and YPD + 100 mM $CaCl_2$ + 3 μM phytosphingosine (4) to give an OD_{600} of about 0.1 after a 16 hour incubation. Following the 16 hour incubation, aliquots were removed every 2 hours for 6 hours and the OD_{600} was determined after diluting 2-fold with 0.5 M EDTA (pH 8.0). The growth rate was determined from the slope of the line formed by plotting the $\ln(OD_{600})$ against time. The 16 hour incubation was included to allow depletion of the sphingolipid, phytosphingosine, or Ca^{2+} that the cells might have accumulated during growth.



1 YPD
2 YPD + 100 mM Ca
3 YPD + 3 μM phytosphingosine
4 YPD + 100 mM Ca + 3 μM phytosphingosine

Figure 20. Acid titration of wild-type and *scs1-1* cells. Cells were titrated as described in Materials & Methods. Wild-type cells were cultured with YPD and *scs1-1* cells were cultured with YPD supplemented with 100 mM Ca^{2+}.


6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
pH
0
50
100
150
200
250
HCl (umol)
scs1−1(P)
scs1−1
wild type(P)
wild type
sorbitol

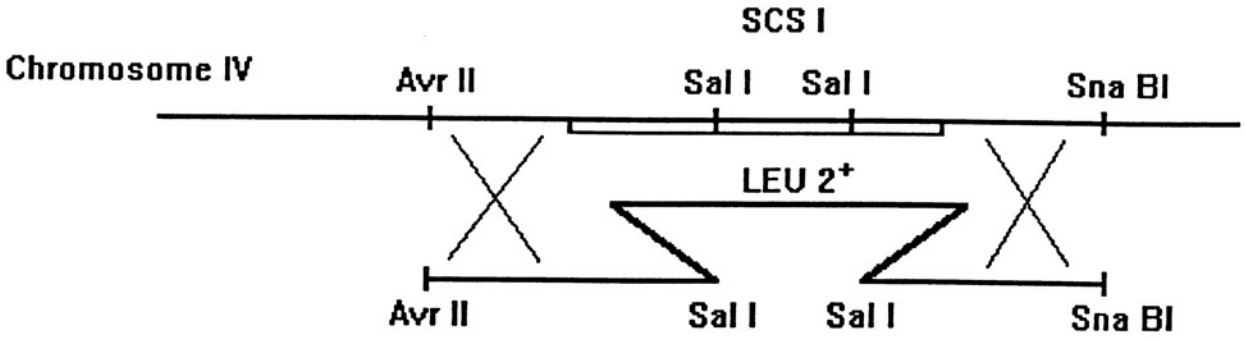
was not buffered by the cell interior. To examine this point directly, wild-type cells cultured with YPD (pH 4.7) and *scs1-1* cells cultured with YPD + 100 mM Ca^{2+} (pH 4.7) were titrated with a strong acid (HCl), and the results were compared with those for cells completely permeabilized by treatment with NaCl-butanol as described in Material and Methods. Both wild-type and *scs1-1* cells treated with NaCl-butanol required significantly more acid to lower their pH to 2.0 than they did without the treatment with NaCl-butanol. The suspending fluid and cells without the treatment with NaCl-butanol gave a similar titration profile, illustrating the relatively minor buffering capacity of the cell, and supporting the idea that the *scs1-1* cells did not have increased proton permeability.

8. Disruption of the *SCS1* gene and determination of serine palmitoyltransferase activity:

To determine whether the *scs1* null mutant is viable, the *SCS1* gene was disrupted by replacing the 908 bp *SalI* fragment (Figure 21) in the *SCS1* coding region with a *LEU2*$^+$ selectable marker giving the *scs1::LEU2*$^+$ allele. Sporulation of the hemizygous diploid (*SCS1/scs1::LEU2*$^+$) followed by tetrad dissection on YPD plates or YPD plates + 25 mM Ca^{2+} or YPD plates + 100 mM Mg^{2+} showed a 2:2 lethal phenotype. All viable spores were leucine auxotrophs, indicating that these spores contain *SCS1*. All spores inheriting the *scs1::LEU2*$^+$ allele died, showing that *SCS1* is an essential gene in yeast. Unlike the spontaneous *scs1* mutants that arise as suppressors of *csg2*, the *scs1* null mutant was not rescued by high Ca^{2+} concentration.

Figure 21. Construction of *scs1::LEU2*$^+$ null mutant. The disruption of the *SCS1* gene was confirmed by Southern blot analysis.

SCS I::LEU2^{+} Knockout Strategy:



Dissected Plates	leu 2^{-}		LEU 2^{+}
YPD	2	:	0
YPD+100mM Mg	2	:	0
YPD+25uM phytosphingosine	2	:	2
YPD + 25mM Ca	2	:	0

However the *scs1::LEU2*$^+$ spores did form colonies when the dissection was done on YPD plates containing 25 μM phytosphingosine.

The disruption of *SCS1* gene by the *LEU2* gene was confirmed by Southern blot analysis [37]. Total yeast genomic DNA from wild type and four *scs1Δ* strains was prepared. After digestion with *SalI*, the DNAs were separated by electrophoresis on a 1% agarose gel (figure 22A). Lane 1, 2, 3, 4 and 5 are the genomic DNA of wild type and four *scs1Δ* strains (named as *scs1Δ*6, *scs1Δ*20, *scs1Δ*21, *scs1Δ*23) respectively. Lane 6 and 7 are the pure 2,200 bp *LEU2*$^+$ (0.1 ng in lane 6 and 1.0 ng in lane 7) fragment that was inserted into the *SCS1* gene and are used as positive controls. After transfer to nitrocellulose, the blots were probed with the 2,200 bp *LEU2* fragment. Band A corresponds to the fragment carrying the *LEU2* in yeast genome. All strains display this band. Band B represents the *LEU2*$^+$ fragment that was inserted into *SCS1* locus. The four *scs1Δ* strains had this band, but the wild type did not. A similar analysis was performed using the *SCS1 SalI* fragment as a probe (Figure 22B). Lanes 1-5 are the wild-type and four *scs1Δ* strains in the same order as in Figure 22A and lanes 6 and 7 are the pure 912 bp *SalI* fragment (0.1 ng in lane 6 and 1.0 ng in lane 7) that was deleted in *scs1Δ* cells. Only the wild type cells had the 912 bp *SalI* fragment at the *SCS1* locus. The Southern blot data confirmed that the *SCS1* gene was knocked out in each of the *scs1Δ* isolates.

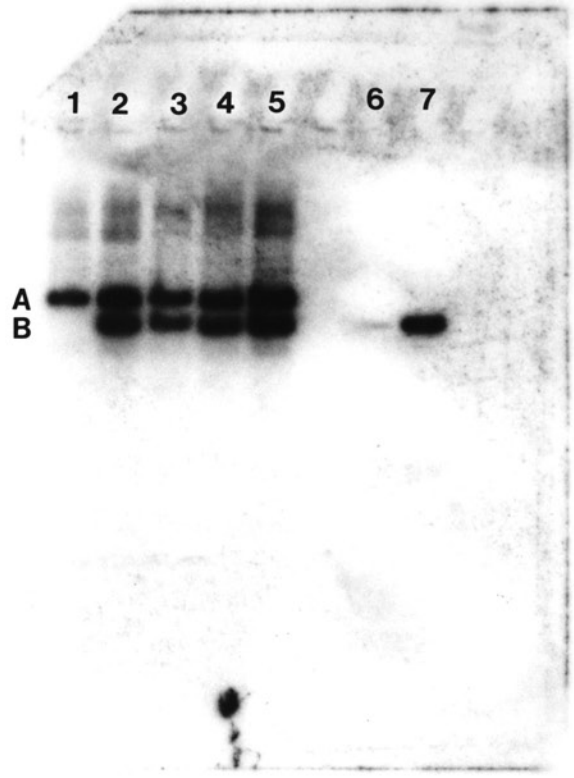
Serine palmitoyltransferase activity was measured in membranes isolated from wild type, *scs1-1* and *scs1::LEU2*$^+$ null mutant cells (Figure 23). The SPT activity of *scs1-1* at early time was reduced relative to that of wild type, and the null mutant had

Figure 22. Southern blot analysis of *scs1::LEU2*$^+$ knockout strain.

A. Yeast genomic DNA was extracted as described under "Material & Methods". Before loading, DNAs were cut with *SalI*. Lane 1 contained wild-type strain; lanes 2-5 are *scs1* Δ 6, *scs1* Δ20, *scs1* Δ21, and *scs1* Δ23 strains respectively; lanes 6 and 7 were loaded with 0.1 ng and 1.0 ng of the purified *SalI* fragment containing *LEU2*$^+$ gene (2200 bp). Probe was the 2200 bp *LEU2*$^+$ segment. Band A is the chromosomal *LEU2* gene fragment and band B is the *LEU2* fragment inserted into *SCS1* locus.

B. The same as "A" except that the probe was 900 bp *SalI* fragment from *SCS1* coding region and lanes 6 and 7 were loaded with 0.1 ng and 1.0 ng of the purified 900 bp *SalI* fragment respectively.

Panel A



Panel B

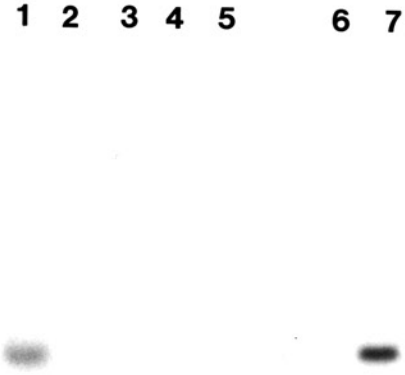
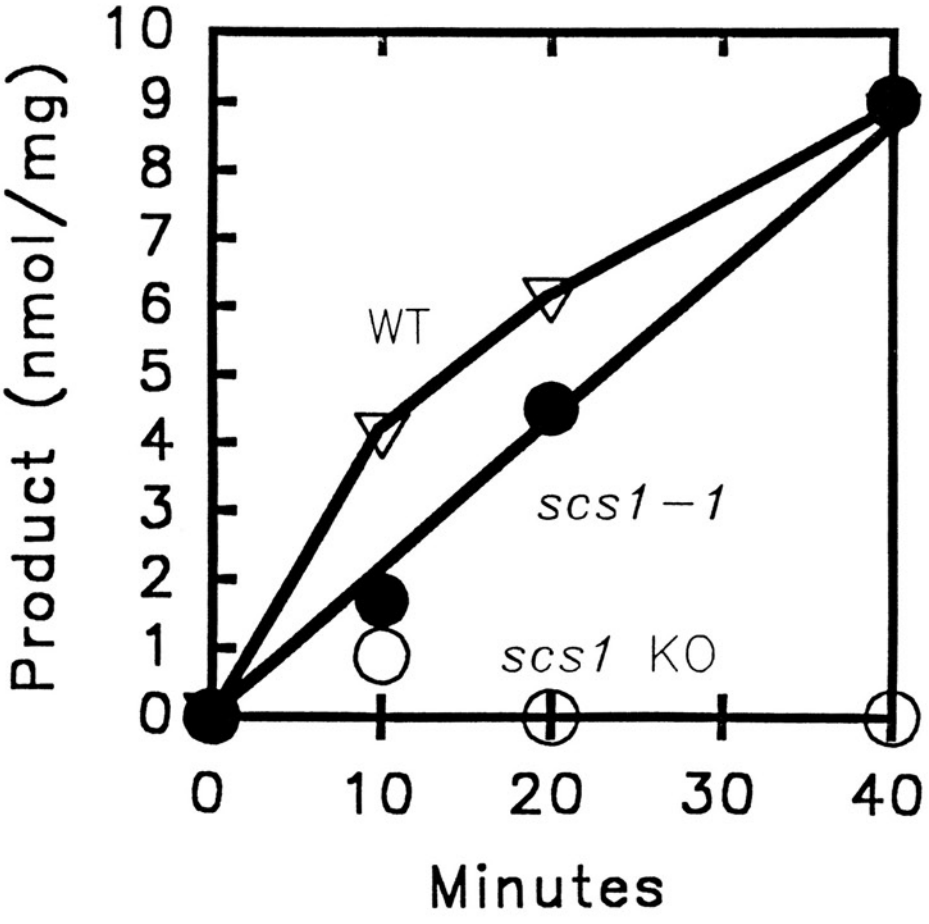


Figure 23. Serine palmitoyltransferase activity in wild type, *scs1-1* and *scs1* null mutant cells. Membranes isolated from *scs1-1 csg2, scs1::LEU2*$^{\pm}$ *CSG2*, and *SCS1 CSG2* cells were assayed for serine palmitoyltransferase activity. The membrane preparation and assay were carried out as described under Materials & Methods.


Product (nmol/mg)
10
9
8
7
6
5
4
3
2
1
0
WT
scs1 −1
scs1 KO
0
10
20
30
40
Minutes

no activity, confirming that SCS1p is required for serine palmitoyltransferase activity. It is interesting to note that serine palmitoyltransferase is activated 2-fold by 0.2 μM Ca^{2+} *in vitro* (Table V), suggesting that some of the Ca^{2+}-related phenotypes may be caused by Ca^{2+}-induced changes in serine palmitoyltransferase activity.

9. Effect of *scs* mutants on the synthesis of inositol-containing sphingolipids:

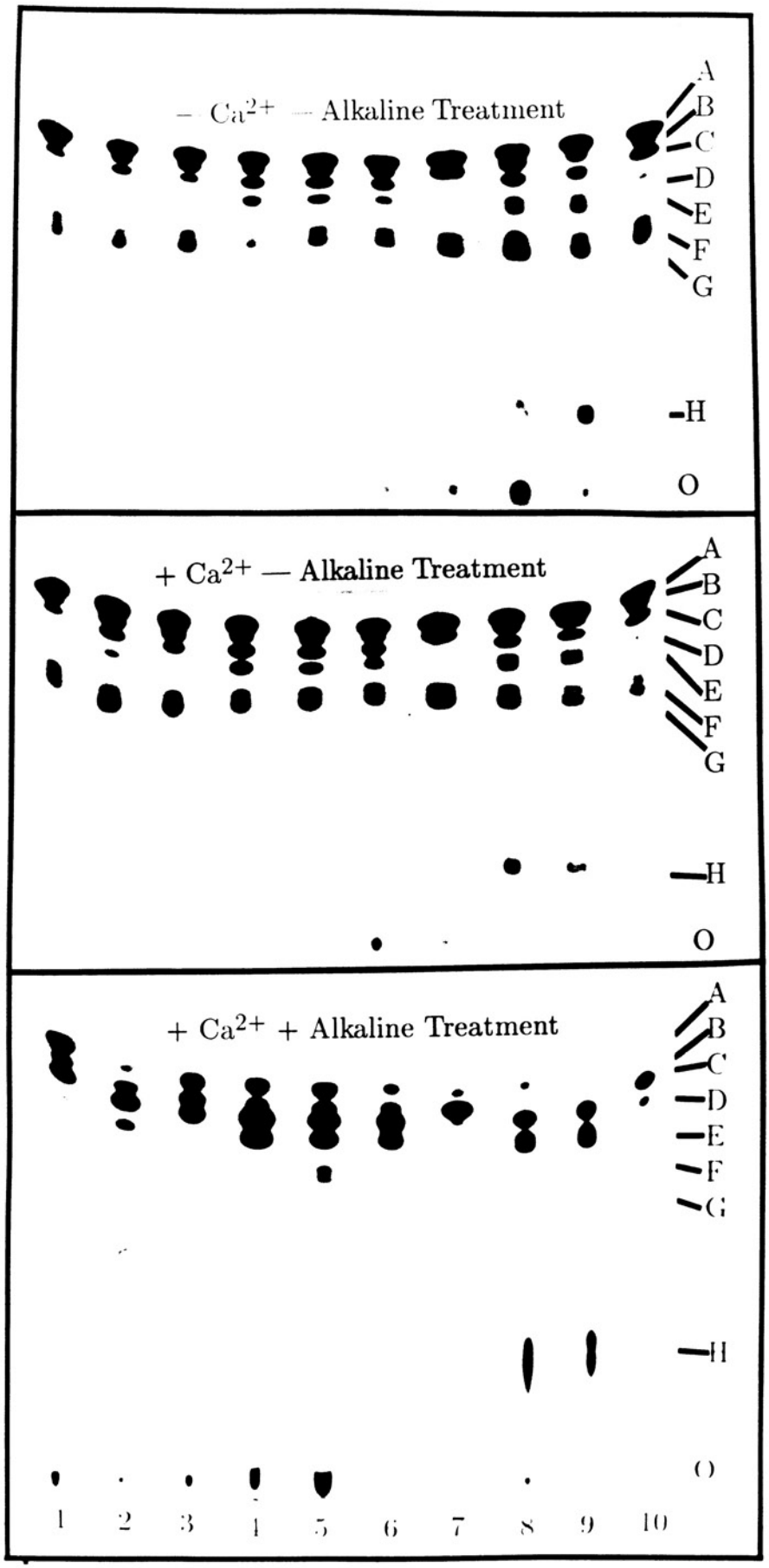
The reversal of the Ca^{2+}-requiring *scs1* and Ca^{2+}-sensitive *csg2* phenotypes by phytosphingosine suggests that the growth defects in these strains are related to alterations in sphingolipid metabolism. This also raises the possibility that other *scs* mutants have altered sphingolipid synthesis. As noted earlier, the major sphingolipids in yeast are inositolphosphorylphytoceramide (IPC), mannoseinositolphosphoryl-phytoceramide (MIPC) and mannose(inositolphosphoryl)$_2$phytoceramide (M(IP)$_2$C). The synthesis of these sphingolipids was measured by labeling cells with [^{3}H]-inositol, extracting the lipids, and separating the different sphingolipid species by thin layer chromatography followed by autoradiographic detection (Figure 24). A distinguishing feature of the inositol phospholipids of *S. cerevisiae* is their stability to deacylation by mild alkaline methanolysis. In contrast to the phospholipids, the phosphoglycerides are deacylated by alkali treatment. The lipids on the top panel were not treated with alkali but they were treated with alkali on the bottom panel. In the wild type cells (lane 8) and in the *scs1* null mutant cells grown in phytosphingosine (lane 9), a characteristic pattern of inositol-containing lipids was observed [Smith and Lester, 1974]. These patterns include phosphatidylinositol (bands A and B),

Table V. Effect of Ca^{2+} on the activity of serine palmitoyltransferase. Membranes were isolated from wild-type cells and were assayed for serine palmitoyltransferase activity, as described under Materials & Methods. The total Ca^{2+} concentration refers to that in the assay solution.

The Effect of Calcium on SPT Activity

Total [Ca] (mM)	Specific Activity (pmol/mg-min)
0	352.5
0.5	868.6 (2.5 fold)
1.1	766.5 (2.2 fold)

Figure 24. Effect of *csg2* and *scs* mutations on the synthesis of inositol-containing sphingolipids. [^{3}H]-inositol labeled sphingolipids synthesized by wild type (lane 8), *csg2::LEU2*$^{\pm}$ (lane 10), *scs1::LEU2*$^{\pm}$ grown in phytosphingosine (lane 9) and *scs1-scs7* (lane 1-7) cells were extracted and separated by silica gel thin layer chromatography, as described under Materials & Methods. Bands A and B correspond to phosphatidylinositol. Bands C and D indicate the position of IPC. Band E indicates the position of MIPC. Bands F and G correspond to lysophosphatidylinositols and band H indicates the position of M(IP)$_2$C. The O indicates the origin.


– Ca^{2+} — Alkaline Treatment
+ Ca^{2+} — Alkaline Treatment
+ Ca^{2+} + Alkaline Treatment
A
B
C
D
E
F
G
H
O
1
2
3
4
5
6
7
8
9
10

inositolphosphorylphytoceramide (ICP-C, ICP-D) (bands C and D), mannoseinositolphosphorylphytoceramide (band E) and mannose(inositolphosphoryl)$_2$phytoceramide (band H). Bands F and G represent lysophosphatidyl inositol. The difference in the ICP-C and ICP-D lipids may result from different levels of hydroxylation [Lester and Dickson, 1993]. IPC-C is synthesized in the endoplasmic reticulum, while IPC-D is synthesized in the Golgi apparatus [Puoti *et al.*, 1991]. Thus, an alkali-stable sphingolipid species that migrated between phosphatidylinositol and inositolphosphorylphytoceramide (lane 7) may represent a ceramide intermediate with reduced hydroxylation that is not normally observed in wild type but accumulates in some of the mutants.

The *csg2* mutant cells (lane 10) synthesized reduced amounts of IPC-D and failed to synthesize the two mannosylated forms of phytoceramide (bands E and H). None of the suppressors (lanes 1-7) restored the ability of *csg2* cells to synthesize normal levels of MIPC and M(IPC)$_2$C, but phytosphingosine did restore IPC-C, MIPC and M(IPC)$_2$C synthesis by *csg2* (data not shown), indicating that the *csg2* mutation affects sphingolipid biosynthesis at a point before phytoceramide formation. The *scs7csg2* mutant strain made only one form of sphingolipid which migrated to a position between the B and C bands (B', lane 7), and may represent an inositolphosphoryldihydroceramide or inositolphosphorylphytoceramide containing an unhydroxylated C_{26} fatty acid. The *scs7CSG2* strain produced all normal forms of sphingolipids but still accumulated the B' form (data not shown). The *scs4, scs5,* and *scs6* (lane 4-6) strains accumulated larger amounts of the IPC-D. Both *scs3csg2* (lane

3) and *scs3CSG2* mutants (data not shown) contained reduced amount of IPC-D.

Since Ca^{2+} influences the growth phenotypes of the *scs1, scs2* and *csg2* mutant cells, it was of interest to determine whether the synthesis of inositol-containing sphingolipids is altered by Ca^{2+}. Cells labeled with inositol in SD media containing 2.0 mM versus 100 mM $CaCl_2$ showed no difference in the pattern of inositol-containing sphingolipids (Figure 24, top panel versus middle panel).

The effect of 25μM phytosphingosine on the growth rates of the other *scs* (non *scs1*) mutants was measured. The *scs2csg2* and *scs2CSG2* mutants, like the *scs1csg2* and *scs1CSG2* mutants, grow in YPD only if it is supplemented with phytosphingosine or Ca^{2+}. Interestingly, the *scs6* and *scs7* mutants display an "allele-specificity" with regard to the *CSG2* allele in that growth of the *scs7CSG2* and the *scs6CSG2* strains is inhibited by phytosphingosine while growth of the double mutant (*scs6csg2* and *scs7csg2*) strains is not.

CHAPTER FIVE

Discussion

CSG2p is a membrane protein with a potential Ca^{2+}-binding site:

The original goal of this project was to identify genes and proteins important in Ca^{2+} homeostasis. A collection of mutants (*csg* mutants) that lost the ability to grow in 10-100 mM Ca^{2+} was isolated as described in Chapter Two [Beeler *et al.*, 1994]. The wild type gene that complemented the Ca^{2+}-sensitive *csg2* phenotype was cloned. Hydrophobicity analysis of the amino acid sequence indicated that CSG2p contains nine transmembrane segments. A potential signal sequence was found at the amino terminus. These findings indicate that CSG2p is a membrane protein. According to the predicted topological representation of CSG2p, a potential Ca^{2+}-binding domain (EF hand) (Figure 1) is found in the first cytoplasmic loop. The *csg2* mutant overaccumulated Ca^{2+} with properties distinct from the Ca^{2+} accumulated by the vacuole (the major Ca^{2+}-sequestering organelle in the wild type cell). Excess Ca^{2+} accumulated by the *csg2* mutant was exchangeable, was released by A23187 and was not competed for by extracellular Mg^{2+}. Thus we postulate that CSG2p is located in a non-vacuolar organelle (possibly endoplasmic reticulum). Ca^{2+} binding to the putative Ca^{2+}-binding site may regulate CSG2p which may function to regulate Ca^{2+} accumulation by the ER or Golgi. When the extracellular Ca^{2+} concentration is too high, this organelle may accumulate lethal amounts of Ca^{2+}. Death of the *csg2* null mutant cells in high Ca^{2+}-containing media may result from: disruption of vital

cellular processes caused by Ca^{2+} overaccumulation. Alternatively, alterations in the sphingolipid composition may make the plasma membrane unstable to high Ca^{2+} concentration.

The csg2 mutant as well as the suppressors of csg2 have altered sphingolipid metabolism.

The suppression of the *csg2* phenotype by a mutation in serine palmitoyltransferase suggests that *csg2* and other suppressors might have altered sphingolipid metabolism. This conclusion is supported by five other observations. 1) Phytosphingosine reverses the Ca^{2+}-sensitive phenotype of *csg2* mutant cells suggesting that phytoceramide synthesis is reduced in *csg2* mutants. 2) The *csg2* mutant cells synthesize reduced amounts of mannosylated sphingolipids (MIPC and $M(IP)_2C$) as well as one form of inositolphosphorylphytoceramide (IPC-D). 3) Phytosphingosine reverses the block in synthesis of these sphingolipids. 4) Sphingolipid metabolism is altered in *csg2* suppressors. 5) The growth of two suppressor mutants (*scs6* and *scs7*) is inhibited by phytosphingosine when the cells contain wild type *CSG2*, and two of the suppressors (*scs2* and *scs6*) fail to grow in inositol-free SD medium if the cells contain wild type *CSG2* (data not shown).

Both the Ca^{2+}-sensitive phenotype of *csg2* mutant and the failure to synthesize mannosylated sphingolipids are reversed by addition of dihydrosphingosine or phytosphingosine to the growth medium. The reversal of the *csg2* Ca^{2+}-sensitivity by dihydrosphingosine suggests that the *csg2* defect is early in the sphingolipid biosynthetic pathway. CSG2p may influence either the formation of 3-

ketosphinganine from serine and palmitoyl-CoA or the reduction of 3-ketosphinganine to dihydrosphingosine. Dihydrosphingosine or phytosphingosine could restore normal levels of phytoceramide in the *csg2* mutant by providing a substrate that is missing (or is not delivered to the correct compartment). Alternatively these compounds could regulate an enzyme in the sphingolipid biosynthetic pathway whose activity is altered by CSG2p (eg,, if the *csg2* null mutant accumulated a toxic precursor when the Ca^{2+} concentration is high, phytosphingosine and dihydrosphingosine might prevent this). Since the *csg2* mutant has a normal growth phenotype in YPD medium, mannosylated sphingolipids are not required for cell growth in YPD, suggesting that, at least for vegetative growth, only IPC-C is required. A drastic reduction in the levels of mannosylated ceramides (MIPC and $M(IP)_2C$) was also recently observed for strains disrupted for the *gda1* (Golgi GDPase) gene which is required for protein and lipid mannosylation [Abeijion *et al.*, 1993]. These mutants are also viable. It will be interesting to determine whether the *gda1* disrupted strain is Ca^{2+}-sensitive, and whether the *csg2* mutant shows altered protein mannosylation.

Analysis of the sphingolipids synthesized by the suppressor mutants demonstrate that the suppressor mutants, like the *csg2* mutant, have defects in sphingolipid metabolism. As seen in Figure 24, the *scs* mutants show alterations in the distribution of sphingolipid species. For instance, the *scs7* mutant produces only one form of inositol-containing sphingolipid which appears to be a species of inositolphosphoryldihydroceramide (with altered mobility possibly due to differences

in hydroxylation). The *scs3* mutant synthesizes reduced amount of IPC-D, while *scs4, scs5*, and *scs6* make increased amounts of IPC-D. Although *scs1, scs2, csg2*, and some *scs7* mutant cells have Ca^{2+}-induced growth phenotypes, Ca^{2+} does not significantly effect the distribution of sphingolipid species synthesized by the cells (Figure 24, panel A vs panel B). The *scs2-scs7* mutants will be investigated in the future to identify other genes in the sphingolipid biosynthesis pathway.

SCS1 encodes a subunit of serine palmitoyltransferase

To investigate the function of *CSG2*, bypass suppressors identifying 7 complementation groups (*scs1-scs7*) were analyzed. The wild type gene of one of these complementation groups, *scs1*, was cloned and sequenced. The SCS1p sequence was homologous to a family of enzymes that use pyridoxal phosphate as a cofactor and catalyze the acyl transfer from an acyl-CoA donor to the α-carbon of an amino acid substrate. The following observations indicate that SCS1p is a subunit of serine palmitoyltransferase. (1) The *scs1* null mutant lacks serine palmitoyl-transferase activity. (2) The *scs1* mutants require phytosphingosine for growth in YPD. (3) The encoded protein is about 40% similar to other enzymes that catalyze this class of reactions and contains the consensus sequence for PLP binding including the essential lysine residue.

Lester and co-workers previously identified two genes, *LCB1* and *LCB2*, that are required for serine palmitoyltransferase activity [Wells and Lester, 1983]. LCB1p has sequence that is 23% identical and 47% similar to SCS1p [Buede *et al.*, 1991].

Surprisingly, LCB1p does not contain the pyridoxal phosphate-binding lysine found in the other members of this family. Apparently both *SCS1* and *LCB1* have to be expressed together in order to obtain functional serine palmitoyltransferase since the null mutant of either gene results in no enzyme activity. *SCS1* may encode a catalytic subunit while *LCB1* may encode a regulatory subunit. Alternatively, both subunits may be required to form the active site of the enzyme. The *LCB2* sequence is identical to that of SCS1p (Robert Lester, personal communication). Although the *scs1* and *scs2* mutants have similar phenotypes, *LCB1* does not complement *scs2* (data not show), therefore *scs2* is nonallelic with *LCB1*.

Since the activity of the serine palmitoyltransferase in *scs1-1* mutants is reduced about 2-fold compared to wild type, and the Ca^{2+} requirement for growth is reversed by phytosphingosine, the *csg2* phenotype is apparently reversed by reducing the flux through the sphingolipid synthetic pathway (possibly by blocking accumulation of a toxic intermediate). While reduced sphingolipid synthesis in the *scs1-1* mutant apparently compensates the *csg2* mutant defect, the reduced rate of synthesis may lead to defective membranes such that *scs1-1* mutants require Ca^{2+} to maintain membrane integrity. Nongrowing *scs1* mutant cells require Ca^{2+} for viability. It would be tempting to speculate that phytosphingosine reverses the *csg2* phenotype by inhibiting the activity of serine palmitoyltransferase since Mandon *et al.*[1991] measured decreased serine palmitoyltransferase activity in neuronal cells in response to addition of sphingolipid bases. However, Lester and coworkers measured no inhibition of the yeast serine palmitoyltransferase (*in vivo* or *in vitro*) by

phytosphingosine [Pinto *et al.*, 1992a]. The antifungal agents sphingofungin B and sphingofungin C are specific inhibitors of serine palmitoyltransferase that are active against *S. cerevisiae* [Zweerink *et al.*, 1992]. The effect of these agents on the *csg2* phenotype at sublethal doses will be interesting.

Sphingolipid metabolism is apparently either regulated by Ca^{2+} or is required for Ca^{2+} homeostasis in yeast:

In *S. cerevisiae*, Ca^{2+} is considered to play an important role in the protein kinase C regulatory system [Levin *et al.*, 1990] and it is required for cell division [Ida *et al.*, 1990a]. In this study, we found that Ca^{2+} is also involved in sphingolipid biosynthesis. This is the first direct evidence shows that the calcium homeostasis and sphingolipid metabolism interact with each other.

The cytosolic Ca^{2+} concentration in both the wild type and the *csg2::LEU2*$^+$ cells is apparently similar since vacuolar Ca^{2+} accumulation (polyphosphate dependent, Mg^{2+}-inhibitable, and non-exchangeable) is not significantly influenced, and the cytosolic Ca^{2+} concentration as measured with fura 2 is not significantly changed [Beeler *et al.*, 1994]. The increased Ca^{2+} loading of *csg2::LEU2*$^+$ cells must, therefore, be accumulated by an organelle that sequesters Ca^{2+} in an exchangeable form. The same mutant also shows a deficiency in sphingolipid biosynthesis. It lacks MIPC, $M(IP)_2C$ and a decreased amount of the Golgi form of IPC. The reversal of *csg2* Ca^{2+}-sensitive phenotype by phytosphingosine and dihydrosphinganine and the synthesis of a MIPC and $M(IP)_2C$ in the presence of phytosphingosine indicate that

CSG2p is required for sphingolipid synthesis.

The growth phenotype of *scs1* mutants is the reverse of the *csg2* phenotype that they suppress. That is, while *csg2* mutants are sensitive to >10 mM Ca^{2+}, the *scs1* strains require 10 mM Ca^{2+} for growth. The mechanism by which Ca^{2+} permits the *scs1* suppressor mutants to grow is not known; one hypothesis is that sphingolipid metabolism is regulated by Ca^{2+}. We have observed that serine palmitoyltransferase is activated 2-fold by 0.2 μM Ca^{2+} *in vitro*. The *scs2* mutants also have a Ca^{2+}-requiring phenotype and altered sphingolipid metabolism. Cloning the wild-type *SCS2* gene will help to understand better the relationship between Ca^{2+} and sphingolipid metabolism.

Some genes that encode Ca^{2+} regulatory proteins such as calmodulin [Davis *et al.*, 1986], protein kinase C [Levin *et al.*, 1990], and calcineurin [Liu *et al.*, 1991; Kuno *et al.*, 1991] have been cloned. It would be interesting to determine whether any of their mutants can suppress the Ca^{2+}-sensitive phenotype of *csg2*, thereby revealing the role of Ca^{2+} in sphingolipid metabolism.

Hypothesis for the mechanism of how mutant csg2 protein causes the alteration of sphingolipid metabolism and the disturbance of calcium homeostasis:

The *csg2* mutant which has a Ca^{2+}-sensitive phenotype was found to have altered sphingolipid metabolism. Normal the wild-type of sphingolipid synthesis is restored in *csg2* mutant cells transformed with the *CSG2* gene, indicating that CSG2p is involved in sphingolipid metabolism. Since exogenous phytosphingosine and

dihydrosphinganine rescue the growth of *csg2* mutant in YPD + 100 mM Ca^{2+} medium (Fig. 19) and phytosphingosine restores normal sphingolipid synthesis, the CSG2p may be involved in the first two steps in sphingolipid biosynthetic pathway (Fig. 12). The *CSG2* gene is non-essential gene whereas both serine palmitoyltransferase and 3-ketosphinganine reductase are required for cell growth suggesting CSG2p regulates sphingolipid synthesis or is required for normal sphingolipid transport.

Two hypothetic models are proposed here to address the possible function of CSG2p based on the information we have.

Model 1: CSG2 protein is involved in the regulation of either one of the first two steps in sphingolipid metabolism. Mutated csg2p causes the accumulation of toxic intermediate (i.e. 3-ketosphinganine) in high Ca^{2+} media. Phytosphingosine and dihydrosphinganine reverse *csg2* Ca^{2+}-sensitive phenotype. Both phytosphingosine and dihydrosphinganine might bypass the defect cause by *csg2* gene by producing the downstream intermediate. The *csg2Δ* cells cultured in YPD + 3 μM phytosphingosine displayed wild type pattern of inositol-containing sphingolipids. Feedback inhibition of serine palmitoyltransferase activity might reduce the accumulated toxic intermediate. The *scs1* mutation might suppress *csg2* calcium sensitivity by slowing down the flux of sphingolipid biosynthesis to reduce the accumulated toxic intermediate. The requirement of *scs1* for Ca^{2+} in the growth medium might be due to the regulation of sphingolipid synthesis by Ca^{2+}. Phytosphingosine reverses the *scs1* Ca^{2+}-requiring phenotype by offering the downstream intermediate and restoring the

wild-type pattern of inositol-containing sphingolipid from *scs1* mutant. Ca^{2+} overaccumulation in *csg2* might be due to the formation of altered sphingolipid components on plasma membrane. However this model does not explain all observations. The *csg2* mutants have normal amount of IPC-C and reduced amount of IPC-D and mannosylated sphingolipids. To test this model, isotope labeled serine or palmitoylCoA can be used to detect any overaccumulated intermediate.

Model 2: *csg2* mutant blocks the transport pathway of sphingolipid from ER to Golgi. The *csg2* mutants have reduced amount of IPC-D, lacks of mannosylated sphingolipids, a similar phenotype was observed in *sec* mutant with a ER -> Golgi block. Exogenous phytosphingosine may restore sphingolipid synthesis by utilizing a Golgi path for sphingolipid synthesis. The *csg2* Ca^{2+}-sensitive and Ca^{2+} overaccumulation phenotypes might be due to the change in the sphingolipid composition of the plasma membrane which causes cell to become less tolerate to high Ca^{2+} and more permeable to Ca^{2+}. It is not clear why *scs1* would suppress *csg2* Ca^{2+}-sensitive phenotype. Perhaps a block in sphingolipid transport is detrimental because of a change in the ER lipid composition. The model also does not explain why *scs1* acquires the Ca^{2+}-requiring phenotype. Perhaps the altered sphingolipid composition of the plasma membrane requires increased Ca^{2+} to maintain membrane integrity. It would be interesting to compare the *csg2* phenotype with *sec* mutants that have a block in ER -> Golgi transport. The synthesis of phytoceramide from phytosphingosine should be investigated to determine which cellular membrane has this capacity. The ability of phytosphingosine to restore the wild-type pattern of

inositol-containing sphingolipid in *sec* mutants should be tested. The *pmr1* mutants have a block in protein mannosylation. The effect of the *pmr1* mutation on sphingolipid mannosylation should be determined. *PMR1* encodes a Ca^{2+}-ATPase which is believed to pump Ca^{2+} into Golgi. GDPase mutants are defective in mannosylation. To determine if the lack of mannosylation causes the Ca^{2+}-sensitivity of *csg2* mutants, the effect of Ca^{2+} on the growth of GDPase mutants should be tested.

Dissecting the sphingolipid biosynthetic pathway by cloning the genes of scs2-scs7:

Establishing nonpermissive conditions for the *scs* suppressor mutant strains permits the isolation of the wild type *SCS* genes from a genomic bank base on their ability to complement the phenotypes. By testing representatives of each complementation group under different conditions (*e.g.*low Ca^{2+}, other divalent cations, +/-phytosphingosine, +/-cell permeable ceramides, +/-inositol, and at high and low temperatures), nonpermissive growth conditions for at least one representative from each of the *scs* complementation groups have been identified as below:

Mutant Strain	Nonpermissive Condition
scs1	All alleles - low Ca^{2+} concentrations
scs2	All alleles - low Ca^{2+} concentrations
scs3	(*scs3csg2*) 10 μM ceramide-C2
scs4	50 mM strontium

scs6 (*scs6CSG2*) Low inositol or 25 μM phytosphingosine

(*scs6csg2*) 10 μM ceramide-C2

scs7 Some alleles - low Ca^{2+} concentrations

(*scs7CSG2*) 25 μM phytosphingosine

For some of the *scs* mutant alleles, the expression of an associated phenotype depends on the presence of a wild type *CSG2* allele.

Cloning wild type genes of *scs2-scs7* will help to identify the genes and proteins required for sphingolipid biosynthesis. For instance, the *scs7* makes an inositolphosphorylceramide which does not accumulate in wild-type cells. Its increased mobility on silica gel thin layer chromatography (Figure 24, relative to IPC-C) suggests that it is unhydroxylated. Therefore, characterization of the *SCS7* gene may lead to information about the hydroxylation reaction that forms phytoceramide. The nonpermissive growth conditions of *scs* mutants can be used to clone the wild type genes that complement the recessive *scs* mutations if the secondary conditional lethal phenotypes and the primary suppression of Ca^{2+} sensitivity are caused by the same mutation. In the event that the associated phenotype is not linked to the suppressing mutation, the wild type genes can also be cloned by screening a genomic library for *scsx csg2* transformants that have reverted to the Ca^{2+} sensitive phenotype.

The epistatic relationship of the *scs* and *csg2* mutants in the sphingolipid biosynthetic pathway can be determined by analyzing double or triple mutants among the *scs* and *csg2* mutations. Change in enzyme activity of the sphingolipid biosynthetic enzymes caused by *scs* mutations is the most direct way of identifying the function

of the *SCS* genes.

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